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THE EFFECT OF METABOLITES ON ANTIMICROBIAL AGENTS[1]

David Gottlieb

SUMMARY

A survey of the literature concerning the effects of a wide variety of toxicants on various bacteria and fungi revealed that the inactivation of these compounds by some metabolic products could be classified into a number of types of detoxication. Among these are 1) chemical combination between the toxicant and one or more metabolites, as in the combination of sulfhydryl compounds with heavy metals or with certain antibiotics; 2) complex formation and chelation of the antimicrobial chemical with products of cellular metabolism; 3) competitive antagonism between a nutrilite and its analogue; 4) pH effects on ionization of the toxicant; 5) oxidation-reduction effects; and 6) enzyme action. One difference between those organisms sensitive to a particular chemical and those resistant to it could be the production of a large amount of a metabolite by the resistant organism; the excess over the metabolic needs could then inactivate the toxic agent.

The effect of metabolites on antimicrobial agents is of common interest to several branches of biology. It is of particular importance to plant pathologists because of their extensive use of toxicants in the control of plant diseases. In this review no attempt has been made to cite the voluminous amount of literature pertaining to the inactivation of toxicants by the products of living cells. Instead, the data in the area have been examined and arranged into a reasonable number of general reactions to explain the mode of inactivation of these deleterious agents. Only a few examples of each type of interaction are given, although many other instances of each kind exist. It is hoped that some of the ideas presented here will suggest another view on the problem of the resistance of certain micro-organisms to various toxic agents.

GENERAL ASPECTS OF METABOLITE-TOXICANT INTERACTIONS.—One of the puzzling aspects of plant disease control is the specificity of many antimicrobial agents for particular pathogens. For example, ferbam will control cedar apple rust whereas captan[2] will not; Karathane[2] controls powdery mildew of apples but neither zineb[2] nor ferbam[2] prevents the disease. Similar situations occur when studies of toxicity are made with pathogens in culture. Sulfur is very effective against *Monilinia fructicola* (Wint.) Honey but is relatively ineffective in inhibiting *Stemphyllium sarcinaeforme* (Cav.) Wilts. One explanation for the specificity of different compounds for certain microorganisms is that toxic effects are due to interference with essential metabolic pathways. Specificity, on this basis, has been difficult to subject to experimental investigation because most of the metabolic pathways that we know are common to all organisms, and further demonstration of this hypothesis must wait until enzymes or metabolic systems unique to the different species of microbes are found. Another interpretation of specificity is based on the fact that living organisms tend to offset the effect of deleterious agents by changing them chemically or even preventing their entrance into the cell. Reactions of this type have been demonstrated in higher animals; benzoic acid is conjugated with glycine, phenols with glucuronic acids, and cysteine with hydrocarbon rings to produce less toxic compounds. In other instances hydrolytic enzymes cleave a noxious material to its less harmful components, or oxidative mechanisms can change a chemical to a more readily tolerated compound. The resistance or susceptibility of microorganisms to toxic agents could then be due in part to the reactions of the various metabolites that the organism produces with such compounds.

The action of a metabolite that is produced by a fungus could increase or it could decrease the toxicity of a chemical. A decrease has been reported more frequently than an increase, but this difference could be misleading since the effects of metabolites on relatively inactive materials are rarely tested. The presence of organic materials of biologic origin often reduces the activity of inhibitory agents (15). Culture filtrates of *Penicillium notatum* Westling, *Bacillus subtilis Cohn* emend. Prasmowsky, and *Streptomyces griseus* (Krainsky) Waks. & Henrici inactivate bactericides (23, 27, 48, 49). Quaternary ammonium compounds and antiseptic dyes are much less active in the presence of the organic materials found in nutrient broth than they are in synthetic media (25, 46). A more detailed analysis of inactivation of a fungicide by culture filtrates of *Aspergillus niger* v. Tiegh. has been presented by Marsh et al (36). When this fungus is grown on fabric treated with copper naphthenate, a bleached zone due to the solubilization of the copper appears on the cloth. The change is caused by the metabolic products of the fungus; if treated cloth is placed in the culture filtrate of the fungus, 52 per cent of the copper is lost to the solution. Individual metabolites can produce similar changes, e.g. loss of copper occurred in the presence of neutral solutions of glycine, aspartic, glutamic, citric, tartaric, and lactic acids. Even the production of acid or of ammonia could cause the loss of copper since these

[1] Accepted for publication July 31, 1956.

[2] Captan is *N*-(trichlormethylthio)-4-cyclohexine-1,2-dicarboximide; Karathane is 2-(methylheptyl)-4,6-dinitrophenyl crotanate and isomers; ferbam is ferric dimethyldithiocarbamate; and zineb is zinc ethylenebis [dithiocarbamate].

agents also decreased the copper content of the cloth.

Diverse types of metabolites are active in reducing toxicity of chemical agents (Table 1). Some of these diminish the inhibitory effects because they form complexes with inorganic ions; others remove the ion from solution by direct chemical bonding. Some can supply an excess of an essential component of a metabolic system for which the fungicide has been a competitive antagonist. Enzymes hydrolyze or in other ways change the compound. Metabolites might increase the toxicity of a chemical by providing orientation centers for concentrating the toxicant on the cell membrane. Changing the pH of a solution also could change the ability of an agent to penetrate the cell membrane by transforming it from an ionized to a nonionized state.

TYPES OF DETOXICATION.—*Chemical combination.*—Chemical reaction between a metabolite and a fungicide or bactericide to produce a new compound that has little or no activity has frequently been reported. The details of such reactions are not usually known; in general, one can only postulate a mechanism for them. When ribonucleic acid is added to a solution of streptomycin, the bacteriostatic effect of the antibiotic is nullified; the 2 compounds unite to form an insoluble precipitate. Not only is the antimicrobial action vitiated, but the properties of the nucleic acid are altered so that this acid can no longer be acted on by ribonuclease (21, 22). Another interesting example is precipitation of inactive compounds of iron by aspergillic acid. This acid is produced by the fungus *Aspergillus flavus* Link (17) and binds iron in an insoluble form to render it inactive. Apparently, chemical combination is responsible for the protective action of spermine or spermidine for bacteria in the presence of *m*-amino-*p*-hydroxyphenyl arsenoxide (39). According to Silverman and Evans (47), an inactive compound is formed when polyamines repress the action of atabrine and growth proceeds. The reversal of the inhibitory action of stilbamidine on Trichomonas by sodium nucleate also is due to direct chemical inactivation (5).

TABLE 1.—*Metabolites that alter the action of antimicrobial agents*

Amino acids	Lactic acid
Cysteine	Acetic acid
Glutamic acid	Tartaric acid
Histidine	
Cystine	*Other N compounds*
Asparagin	Urea
L-Tyrosine	Allantoin
DL-Tryptophan	Spermine
Methionine	Spermidine
Proline	Urethan
Glycine	
Aspartic acid	*Nucleic acids*
Alanine	Nucleic acid
Isoleucine	Thymonucleic acid
DL-Norleucine	Ribonucleic acid
Arginine	
Proline	*Enzymes*
Glutathione	Penicillinase
Purines, pyrimidines	Trypsin
Uracil	Emulsin
Adenine	Amylase
6-Amino purine	Arginase
Hypoxanthine	Bacterial filtrates
	Antibacillin
Phospholipids	
Lecithin	*Fatty acids*
Cephalin	Oleic acid
Sphingomyelin	Myristic acid
Growth promoters	Stearic acid
p-Aminobenzoic acid	
Cocarboxylase	*Alkaloids*
Pteroylglutamic acid	Quercitrin
Pantothenic acid	Rutin
Nicotinic acid	
Vitamins K_1 and K_2	*Sugar derivatives*
Organic acids	D-Glucose-6-phosphate
Citric acid	D-Fructose-1,6-phosphate
Pyruvic acid	Glucose
Fumaric acid	Other sugars

The frequency with which the amino acid cysteine reverses the activity of a large number of varied bactericides and fungicides is at first puzzling. Such diverse agents as mercury, copper, arsenic, bismuth, silver, penicillin, streptomycin, clavacin, penicillic acid, acrylphenone, and gliotoxin (2, 10) are detoxified by this amino acid. Often the tripeptide glutathione, which contains cysteine, is also very effective in reversing growth inhibition. This detoxifying property is due to the very active sulfhydryl group of the molecule. Those toxicants that react with the sulfhydryl group in the microorganism (primarily in the enzyme protein) can also react with cysteine. When cysteine is free, it competes for the valences of the toxic material and binds them into a physiologically inocuous compound.

Voegtlin and coworkers (52) studied the trypanocidal effects of an arsenic compound, arsenoxide, and found that when cysteine was injected into mice, it overcame the therapeutic effect of the drug. Later (10), similar results were found for *Treponema pallidum* (Schaud. & Hoff.) Schaud. in culture; arsenoxide's inhibitory properties were reversed by cysteine and glutathione. About 80 times more arsenic was required for the same degree of inhibition in the presence of these agents than in their absence. For mercuric chloride, 16 times the normal inhibitory concentration was necessary, and increases of the same order of magnitude were required for various bismuth compounds. With these organisms, the protecting agent must react with the arsenic before the latter has acted on the cell. Any free drug is instantaneously detoxified. This reaction could be explained as a simple combination in which amino acid successfully competes with the protoplasm for the arsenic. The arsenic is toxic because it combines with the sulfhydryl groups in protoplasm, as in reaction A.

$$\text{(A)}\quad R{-}As{=}O + 2\,SHR' \rightleftharpoons R{-}As(SR')_2 + H_2O$$

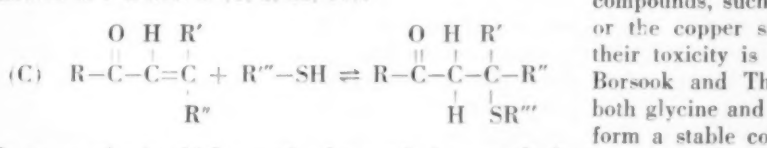
Apparently the same type of reaction takes place with

mercuric chloride. If a "stronger" sulfhydryl compound, such as H_2S, is used, the mercury that is bound on the protoplasm can be removed and growth is resumed. In both instances the metals are thought to combine with the SH groups of the protein of an enzyme according to reaction B with the result that the enzyme is inactivated.

(B) $Hg + 2\ protein{-}SH \rightarrow (protein{-}S)_2Hg + 2H$

Phenyl mercuric nitrate is also ineffective in the presence of cysteine or glutathione (51). Compounds in which the SH group is protected, as in cystine, cannot reverse the inhibition.

Cysteine also can protect an organism against the action of entirely different types of fungicides or bactericides, such as clavacin and penicillic acid. These compounds contain no metal that can be bound by sulfhydryl groups; however, a comparison of a number of organic compounds that are inactivated by cysteine and glutathione reveals that many have an α, β unsaturated ketone in the molecule. The sulfhydryl group of cysteine combines across this linkage as indicated in reaction C (2, 8, 16, 44).



In terms of microbial growth, the result is a marked increase in the concentration of any such agent necessary for the inhibition. To inhibit *Escherichia coli* (Mig.) Cast. & Chalmers a dilution of only 1 g clavacin in 300,000 ml of water was necessary; in the presence of cysteine (20 mg/ml), this was increased to more than 1 g in 10,000 ml or at least 30 times greater (16). With neoarsphenamine, the reaction is presumably across the arsenic-to-arsenic bond; free SH groups disappear and stable nonbactericidal compounds are formed.

The antifungal agent gliotoxin also is inactivated by cysteine. This fungicide contains an S-S bond, and the amino acid might inactivate this compound by reducing the disulfide linkage. With streptomycin, the inactivation is not readily explained on the basis of chemical combination and might be due to other mechanisms of reversal.

Complex formation and chelation.—Destruction of the toxicity of the metallic cation by the formation of a complex or chelate between an amino acid and a toxic heavy metal is another type of reaction that is encountered. An interesting example of this detoxification is given by Schade (45), who used cobalt to prevent the growth of *Proteus vulgaris* Hauser. This cation was toxic to the bacterium at 0.25 μg per ml in a chemically defined medium but was relatively inocuous in nutrient broth. The detoxication of cobalt was traced to 2 amino acids, L-histidine and L-cysteine. If either of these acids was added in proper amounts to a chemically defined medium, growth proceeded regularly even in the presence of the metal. The molar ratio for this reversal was 2:1 of the amino acid and metal, respectively. The toxicity of cobalt could be reversed even after many hours' contact with the cells, indicating that the bond of cobalt with the cell is less tenacious than that with the amino acid. The sequence of addition had no effect on the final result. Two molecules of histidine combine with 1 molecule of cobalt to give 1 molecule of cobalt dihistidine and 2 hydrogen ions. In the presence of oxygen, 2 of these combine to form a molecule of oxy-bis-(cobalto-dihistidine). This then changes to an irreversibly oxygenated form (Reaction D).

(D) $2\ Histidine + Co^{++} \rightleftharpoons Co\ (histidine)_2 + 2H^+$

$2\ Co\ (histidine)_2 + O_2 \rightleftharpoons [Co\ (histidine)_2]\ O_2$

The reversal of cobalt inhibition by cysteine can also be due to a combination of cysteine with the metal ion. Such complexes have been demonstrated for both cobalt and nickel (38).

Some of the most common fungicides contain copper in various forms. Often these are applied as insoluble compounds, such as cuprous oxide, copper carbonate, or the copper salt of fatty acids, however, most of their toxicity is due to the formation of copper ion. Borsook and Thimann (7) have demonstrated that both glycine and alanine will react with cupric ions to form a stable complex. Two molecules of the amino acid will link with 1 copper ion without involving primary valency bonds. Four different complexes may be formed, depending on the hydrogen ion concentration. Such reversal of copper toxicity has been recently demonstrated biologically (43). *Mycobacterium phlei* Lehmann & Newmann is very sensitive to copper; the addition of L-glutamic acid will offset this effect and allow growth to proceed normally. Similar results were obtained with sodium citrate, and these too have been attributed to the formation of nonionizable complexes.

Another interesting example of inactivation of a toxic agent by metabolites has been reported by Zentmyer and Rich (57). The toxicity of copper-8-quinolinolate to spores of *Aspergillus niger* and to the mycelium of *Botryosphaeria ribis* Grossenb. & Duggar was reversed by the presence of casein hydrolysate, of L-histidine, or of L-cysteine but not by L-methionine, L-glycine, or L-cystine. Cysteine was especially effective in removing the copper from the quinolinolate, producing the copper amino acid chelate and free quinolinol.

Competitive antagonism.—Another mechanism by which a large number of metabolic substances can nullify the action of a germicide is competitive antagonism. A chemical compound that is structurally similar to a coenzyme, substrate, or other essential component of an enzyme system can attach itself to the positions in or on the cell that normally are occupied by the metabolite and thereby prevent the cell functions. Some organic fungicides or bactericides might play this role for as yet unknown essential metabo-

lites. Just this approach led Woods and Fildes (55) to the discovery of *p*-aminobenzoic acid. They found that extracts from streptococci and from yeasts antagonized the inhibition of bacteria by the potent bactericide, sulfanilamide. An extensive search of compounds structurally related to this bacteristat and having a similar antagonistic action resulted in finding the until then unknown metabolite, *p*-aminobenzoic acid. On the basis of competitive antagonism, many compounds have been synthesized and found to be good bactericides. This relationship between the essential metabolite and its analogue is readily apparent in a number of instances. Sulfanilamide, for example, resembles *p*-aminobenzoic acid (Fig. 1) and is an effective inhibiting agent. Many organisms require pantothenic acid for growth; pantoyltaurine, a very closely related compound in which the COOH group is replaced by SO_3H, is a competitive analogue and inhibits growth (33). Similarly, pyridine-3-sulfonic acid is an effective bactericide because of its structural relationship with nicotinic acid (34). In each of these examples the addition of the normal metabolite overcomes the effect of the inhibitor.

Supplying the end product.—There is another type of antagonism, noncompetitive interference, in which naturally occurring metabolites take part. In this case, the reversing agent is not structurally related to the germicide, nor does a constant ratio exist between the initial growth inhibitor and the metabolite. The addition of the metabolite merely supplies the end product of the reaction or chain of reactions with which the inhibitor interfered. This type of nullifying effect occurs in the reversal by methionine of the inhibition of *E. coli* with 2-chloro-4-aminobenzoic acid (CABA) (54). Using methionine labelled with C^{14} in the omega carbon, Wang and his associates showed that the methionine, which was supplied in the medium, had diffused into the cells and had taken the place of the similar amino acid that would have been produced in the absence of CABA. The complete protein that was necessary for growth could then be formed. Another interesting example of such antagonism is the inhibiting effect of indoleacrylic acid on the growth of *E. coli* and of *Salmonella typhosa* (Zopf) White (13). The deleterious effect of this compound can be overcome by the presence of the amino acid, tryptophan. In the presence of tryptophan and indoleacrylic acid, the growth of *S. typhosa* is proportional to the amount of the amino acid that is added. Idoleacrylic acid is closely related to tryptophan and prevents the formation of this amino acid but does not prevent its utilization. Unlike competitive antagonism, the metabolite offsets the inhibition by supplying the end product of a reaction.

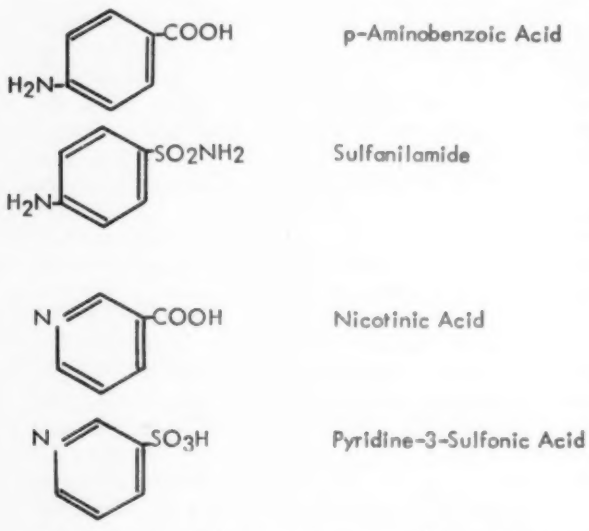


FIG. 1. Structural formulae of some metabolites and their analogues.

One can perhaps ascribe a similar action to the reversal by glutamic acid of the inhibition of the yeast, *Saccharomyces cerevisiae* Meyer, with phenylpantothenone (56). Histidine is also active in overcoming the effect of phenylpantothenone, and some other amino acids are less effective. This specific reversal was based on the original observation that natural products overcome the bactericidal effect of panthothenone. The same explanation might account for the observation that some of the amino acids reverse the inhibition of bacteria by furacin, phenyl (5-nitro-2-furaldehyde semicarbazone) (20); alanine, glutamic acid, lysine, and isoleucine antagonize the toxic action. References to the reversal of toxic action by purines and pyrimidines, such as in the reversal of the protective action of sulfanilamide by adenine in mice infected with *Micrococcus pyogenes* var. *aureus* (Rosenbach) Zopf, are common (12, 37). Probably the sulfur compound interferes with the normal synthesis of adenine by *M. aureus;* the presence of excess adenine then allows normal metabolism to proceed.

Hydrogen ion concentration effects.—The ability of an organism to produce substances that change the pH of a medium is well known. Some of these, such as citric, lactic, and oxalic acids, lower the pH; others, such as ammonia or organic amines, raise it. In addition, the differential absorption of inorganic ions can bring on similar transformations in the acidity of a medium. Basic compounds tend to have their ionization suppressed in basic solution, and acid compounds are affected the same way in acid solutions. Often it is only the nonionized form of an organic molecule that readily passes through the cell membrane. Propamidine, a highly basic compound, inhibits growth of fungi, bacteria, and protozoa much more in basic than in acid media (5). In highly acid media, the cationic form appears, whereas under basic conditions the molecule is nonionized and carries no charge. Under acid conditions, the nontoxic hydrogen ion competes with the positively charged propamidine ion for a position on the cell membrane (11). Atabrine, another highly basic compound, is also more effective

under basic conditions in which the molecule is relatively undisassociated (47).

Hydrogen ion concentration also determines germicidal effects by affecting the solubility of compounds, as shown with copper napthenate. One would expect even more marked effects on such compounds as cuprous oxide, in which even small amounts of acid could cause large solubility changes. The bacteriostatic action of streptomycin can be reversed by changing the pH of the milieu to pH 4–5 with sulfuric acid if no more than 4 hours has elapsed (31). Longer contact with streptomycin has an irreversible effect. This detoxication is not due to the destruction of streptomycin for this material is stable at high acidities.

Oxidation-reduction effects.—Very few instances of reversal due to this type of mechanism have been clearly established. McIlwain (35) postulates, however, that overcoming of inhibitory effects of iodinin on streptococci by vitamins $K_1 + K_2$ could take place through a reduction of the iodinin compound by these metabolites. Not only these naturally occurring materials but other similar highly oxidized compounds, such as anthroquinones and naphthoquinones, will also protect the organism from iodinin (Fig. 2).

The reduced activity of streptomycin in the presence of ascorbic acid or of cysteine has also been attributed to oxidation-reduction processes (26).

Enzyme effects.—The role of enzymes as catalysts for cellular reactions is well known, and almost any reaction studied is presumed to be under enzymatic control. With such a wide variety of enzymes, some with a narrow range of substrates and others with a wide range of substrates of similar configuration, it is also reasonable to assume that the structure of a germicide might be related to one of the normal substrates of these enzymes. Compounds might either become more toxic if they are hydrolyzed by the enzymes to release a toxic moiety, or they might lose toxicity because of degradative changes in the molecule.

The effect of enzymes on germicides can be seen readily in the production of penicillinase by various bacteria. After the initial observation by Abraham and Chain (1) that *Escherichia coli* was not inhibited by the antibiotic, extracts of *E. coli* were made that inhibited the action of penicillin. Further studies disclosed that an enzyme, penicillinase, was involved (41). Penicillinase apparently is not a single compound, for many bacteria produce different enzymes that inactivate this antibiotic. They remain inside the cell in some cultures and diffuse out of the cell in others. The ability of some organisms to withstand the antibiotic could be related to the production of this enzyme. In one study, 23 penicillinase-producing organisms were tested for their susceptibility to the antibiotic (5). Twenty-one of these strains were not inhibited, whereas 2 of them were inhibited by relatively high concentrations. Production of the enzyme is not the only factor in such resistance, since offspring of strains that were originally sensitive became resistant following serial transfers on media containing the antibiotic, yet these resistant strains produced no penicillinase.

Not only specific enzymes but even more general ones, such as amylase, have been shown to inactivate penicillin (29). Furthermore, the proteases, many of which are commonly produced by microorganisms, will split germicides that are "peptide-like." Subtilin, an antibiotic effective in seed treatment, is largely inactivitated by pepsin, trypsin, pancreatin, or erepsin (32). Lipases can inactivate some germicides that are esters of fatty acids. When circulin and polymixin B were acted on by lipase, from 60 to 90 per cent inactivation occurred. Since these polypeptide-like compounds contain an acid similar to pelargonic acid, the inactivation is presumed to be due to the splitting off of this acid, a reaction that takes place readily on acid hydrolysis (42).

A possible enzymatic inactivation of a bactericide might occur also with the polyamine, canavanine, which inhibits the utilization of arginine. Furthermore, arginase obtained from liver or from jack bean can split canavanine and form canaline (9). Some bacteria, such as *Streptococcus faecalis* Andrewes & Horder, are resistant to the inhibition. Volcani and Snell (53) have suggested that arginine dehydrolase in the resistant streptococcus might inactivate the canavanine by hydrolyzing it into canaline and urea.

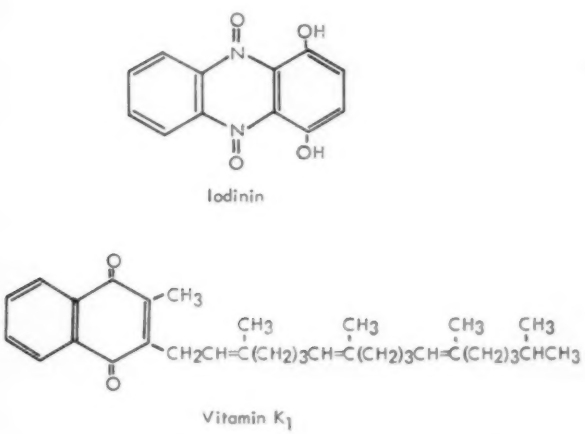
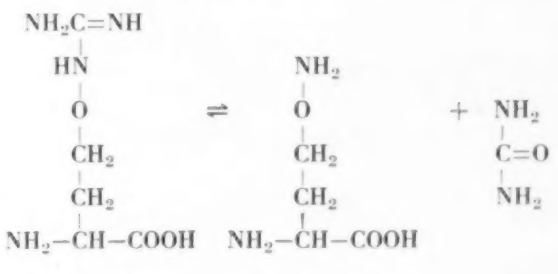


FIG. 2. Structural formula of iodinin and vitamin K_1.



Our own studies have shown that many inhibitory agents, such as actidione, clavacin, and chloramphenicol, are broken down by the microflora of normal soil but remain active in a sterile soil (18, 19). Furthermore, chloramphenicol is hydrolyzed by many bacteria to the inactive components, dichloroacetic acid and *p*-nitrophenylserinol (Fig. 3), and can even be further dissimilated by some bacteria (49, 50). The resulting population consists of an increased proportion of resistant organisms.

The presence of enzymes that hydrolyze glycosidic linkages might also play a role in such processes. Amygdalin, for example, is readily hydrolyzed by β-glucosidases to release the toxic material HCN (Fig. 3). The presence of such an enzyme in one fungus could bring about its death, whereas another fungus that did not produce this enzyme might survive. β-Glucosidases are produced by many wood-inhabiting fungi as well as plant pathogenic fungi.

Proximity effects.—Of a different nature from the reactions discussed above are "proximity effects." In these instances, it is the locus on or in the cell at which the inhibitory agent is to act that is altered. If the forces at the locus are oriented for attracting the fungicide, then its resultant activity is increased; if they are not so disposed, the converse occurs. Such reactions can account for the effect of fatty acids of from 10 to 16 carbons. The activity of streptomycin is enhanced by these fatty acids, whereas that of penicillin is weakened (3). The activity of another strongly basic material, 9-amino acridine, is also enhanced. With cetyldimethylbenzylammoniumchloride, a basic bactericide, there is an unexpected decrease in activity, but in this case the fatty acid combines with the quaternary amine before it has been adsorbed on the cell. A theory for these phenomena is that the fatty acids are bound to the lipids of the cell by van der Waal bonds. This increases the concentration of negative groups near the loci of antibacterial activity, thereby exerting a stronger attraction for basic materials. Basic inhibitors, such as streptomycin and 9-aminoacridine, are attracted in greater concentration; negatively charged materials, such as penicillin, tend to be repulsed from such charged centers. Neutral materials, naturally, are unaffected.

Lecithin effects.—Lecithin is a compound, or mixture of compounds, composed of fatty acid esters of glycerol in which the glycerol is linked also to phosphorylated choline. It is produced by bacteria and fungi as well as by many other plants and animals. Since it is a detergent, its protective activity is surprising, since surface active compounds usually increase bactericidal activity. Yet lecithin protects dermatophytic fungi against the fungicidal activity of propamidine (11). One clue to the reversal of propamidine toxicity is the resemblance of lecithin to quaternary ammonium compounds; both are cationic because of the quaternary ammonium ion. Since lecithin nitrogen has a positive charge, it competes with propamidine for the anionic position on the bacterial cell. Lecithin, which is not toxic, thus prevents the more toxic base propamidine from being adsorbed on the cell surface (14). A similar result is evident in the protection of *Micrococcus tetragenus* Gaffky against synthetic detergents in the presence of various lipoids. Baker et al (4) used oxygen uptake as a criterion. Cephalin, sphingomyelin, and lecithin, among others, prevented the inhibition of oxygen uptake. A simple ionic exchange,

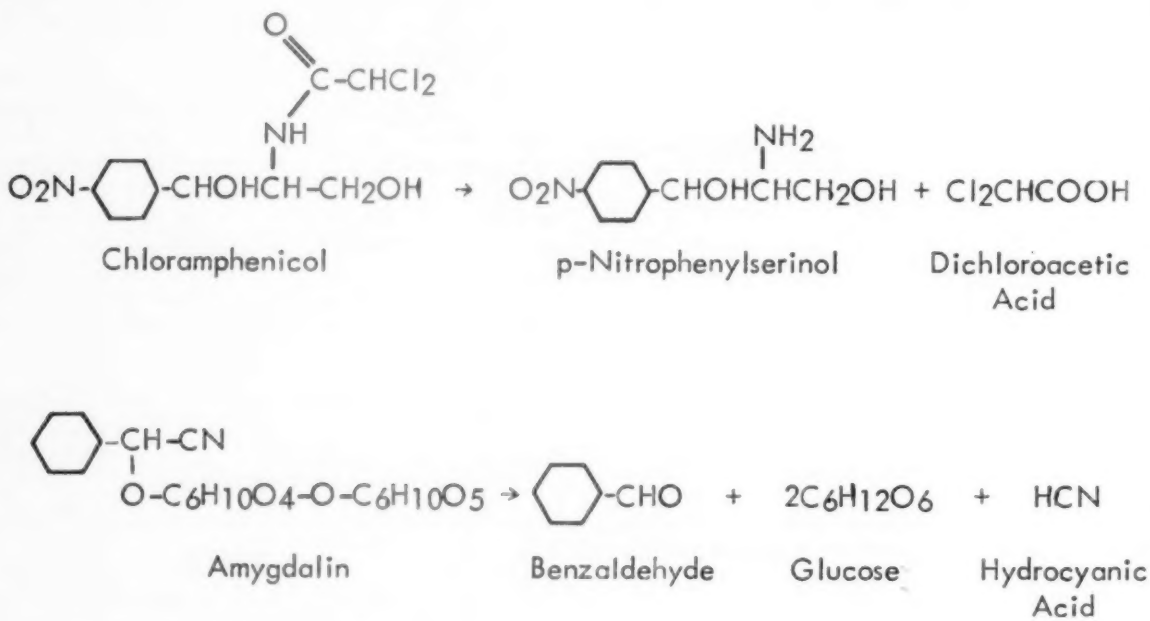
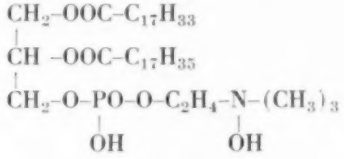


FIG. 3. Effect of microbial enzymes on antimicrobial agents.

however, will not adequately explain such results, for the materials reverse the effects of anionic as well as cationic detergents.



Oleyl-stearyl-lecithin

In other instances, the action of phospholipids is not readily understood. Thus, phospholipids, especially cephalin, inhibit the action of tyrothricin (40). This is a polypeptide that bears no resemblance to the molecule that inhibits its action. Similar protective effects of lecithin for fungi are reported when hexylresorcinol is used to inhibit growth (30).

Displacement effects.—Another mechanism of detoxication is the displacement of a toxic agent by a relatively innocuous compound, such as the reaction of atabrine or of quinine in the presence of long chain polyamines. Both spermine and spermidine nullify the inhibition of *E. coli* by atabrine or by quinine (39). Apparently the more basic polyamines displace the toxic material, atabrine, from the negatively charged position on the cell. If this is so, why is not spermine, which is occupying the same position on the cell, toxic? Two factors are necessary for toxicity. First, the compound must be innately toxic to some cell mechanism; second, it must be in the correct position to exert this effect. Atabrine and quinine satisfy both criteria, but spermine fulfills only the requirement of position.

A model for this viewpoint of displacement is available from studies on the adsorption of basic materials by clay colloids. Basic dyes such as methylene blue, malachite green, and janus green are readily adsorbed by the negatively charged colloids and are rather tightly bound so that they cannot be readily replaced by hydrogen or other cations. If the adsorption complex is treated with the base brucine, some of the dye is displaced. The basic materials need not be permanently fixed on the colloid, for thermal activation is always present and the materials are going on and off the negatively charged positions to produce an equilibrium. In such a system, one can readily picture that the introduction of another basic compound could occupy some of the positions that are temporarily free.

DISCUSSION.—An evaluation of the evidence presented here leaves no doubt about the ability of diverse metabolic products to influence the antimicrobial activity of various chemical agents used in disease control. That such materials function in this way in lower organisms must be substantiated by more definitive studies. Investigations of the resistance of bacteria to sulfonamides indicate that some of the resistant strains produce more *p*-aminobenzoic acid than do susceptible ones (28). Furthermore, strains of *Micrococcus pyogenes* var. *aureus* that were sensitive to sulfanilamide could be made resistant to this compound. The resistant offspring produced 60 times more *p*-aminobenzoic acid than did the sensitive parents. Thus in certain types of resistance, there is a correlation between the presence of a metabolite produced by the living cell and its defense against deleterious agents. This type of response is one of the factors in the adaptation of house flies to DDT. Probably, adaptation plays only a minor role in the specificities to germicides, except where a population has been exposed to such materials for many generations.

One cannot assume, however, that all increase in apparent resistance of an organism on the addition of a metabolite is due to a specific detoxication. The additive could function merely to produce better conditions for growth. Under more favorable conditions, more cells are produced and the amount of toxicant necessary to inhibit growth must be greater. Arginine, for example, reduces the action of furacin only because it stimulates growth (20).

The various defense mechanisms are usually purely fortuitous and depend on the variation in normal physiological activities of a particular species or strain. The organism that contains high concentrations of some of the amino acids free in the cytoplasm, or even sufficient amounts to diffuse out of the cell, would tend to be more resistant than another that had little in excess of the requirement for protein synthesis. Similarly, the microbe that produces citric acid could be relatively insensitive to certain chemical agents because it forms complexes or chelates with the toxicant. If the toxic material resembled a normal substrate for an enzyme system in one species, then it would be inactivated by this process and the microbe would be resistant. The accumulation of phospholipids near the surface of a microbe, as in the gram-negative bacteria, could be responsible for the resistance of many of these organisms to some antibiotics. Though the results that are obtained in culture tend to be magnified, they at least indicate that such defence mechanisms exist.

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ERADICANT TREATMENTS FOR NARCISSUS BULBS AND GLADIOLUS CORMS HARBORING SOYBEAN NEMATODE CYSTS [1]

N. N. Winstead and C. B. Skotland[2]

SUMMARY

Several treatments, including eradicant treatments for the bulb and stem and the root-knot nematodes and a prestorage treatment for the control of Fusarium bulb and corm rots, were found lethal to the larvae and eggs in cysts of the soybean nematode. No reproduction occurred on soybean roots inoculated with cyst material steeped in hot water at 48–49°C for 30 minutes or at 54–55°C for 15 minutes. A 0.5 per cent formalin treatment at 45°C for 3 hours was also effective; however, this treatment at room temperature was not effective. Soak treatments with sodium 2, 4,5-trichlorophenoxide at a concentration of 3 lb. of an 85 per cent formulation to 100 gal. of water for 15 minutes or 2 lb. to 100 gal. of water for 1 to 2 hours was also lethal to the eggs and larvae in cysts of the soybean nematode. Two additional bulb and corm treatment materials, 97.5 per cent phenylmercury acetate and 5 per cent ethylmercury phosphate, were not effective.

The soybean cyst nematode, *Heterodera glycines* Ichinohe, 1952, was reported in southeastern North Carolina in 1955 (8). This nematode causes a severe disease of soybeans, *Glycine max* (L.) Merrill, known as yellow dwarf or soybean cyst nematode disease. In this infested area, gladiolus (*Gladiolus* spp. Hort.) and narcissus (*Narcissus pseudo-narcissus* L.) are major crops. Although these crops are grown primarily for cut flowers, surplus bulbs are often sold. It has been reported (5) that neither of these crops is a host of *H. glycines*; however, the cysts of this nematode might be disseminated as contaminants on bulbs and corms harvested from infested soil. The present study was undertaken to develop methods of preventing spread of the nematode by this means. A preliminary report has been published (7).

MATERIALS AND METHODS.—Treatment materials included hot water and hot-water-formalin, which have been generally recommended for the control of root-knot nematodes (*Meloidogyne* spp. Goeldi, 1887) and of bulb and stem nematodes (*Ditylenchus* spp. Filipjev, 1936) (2, 3, 4), as well as materials shown to be lethal to contents of cysts of the golden nematode, *H. rostochiensis* Wollenweber, 1923 (1). Other chemicals tested were those shown by F. A. Haasis (personal communication) to be effective in the control of Fusarium rots of gladiolus and narcissus in North Carolina. These included 85 per cent sodium 2,4,5-trichlorophenoxide (sodium trichlorophenate) as Dowicide B; 97.5 per cent phenymercury acetate, as Mersolite W;

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and 5 per cent ethylmercury phosphate, as New Improved Ceresan. Concentrations were based on quantities of commercial formulations rather than on quantities of active ingredients.

Soil containing cysts was collected from a heavily infested field and screened through both 20- and 80-mesh sieves. The cysts and debris collected on the 80-mesh sieve were divided into 5-g lots and placed in small muslin bags. Each bag contained more than 1500 cysts.

The variety of narcissus used in these tests was Helios and varieties of gladiolus were Snow Princess and Picardy. Fifty bulbs or corms of each of these varieties and the cyst material were soaked in the various treatment solutions. No bulbs or corms received the 54–55°C hot-water treatment. DuPont spreader-sticker (principal functioning agents—sodium sulfates of mixed esters of long-chain alcohols and fatty acids and diethylene glycol abietate) was used at the rate of 1 ml per liter of hot water or of treatment solution. A hot-water bath (Precision Scientific Co.) equipped with a thermoregulator was used to maintain a constant temperature. The temperature dropped when bulbs were added, but the cyst material was added only after the desired temperature was obtained. Treatments were applied to gladiolus corms in January and July as prestorage treatments and to narcissus as prestorage treatments in early July and premarketing treatments in early August. Prestorage treatments were applied to mature bulbs within 2 weeks after harvest. After treatment, bulbs and corms were stored and later planted in the field. Observations were made on rate of emergence, on phytotoxicity, and on yield of marketable flowers.

Treated cyst material was divided into 5 lots of approximately 1 g each. One lot, placed in a Syracuse watch glass and kept moist, was examined microscopically at weekly intervals for 4 weeks for the presence of viable larvae. The remaining lots were placed in steamed soil in 4-in. clay pots, and seeds of the Roanoke soybean variety were planted. Except for the initial experiments, treatments were not randomized. After the soybeans had grown in the greenhouse for 6–8 weeks, the soil in these pots was screened through 20-, 80-, and 325-mesh sieves. The material collected on the 80-mesh sieve was examined for the presence of new females. The material collected on the 325-mesh sieve was placed in a Baermann funnel and males and larvae were recovered. These experiments were repeated 3 times.

EXPERIMENTAL RESULTS.—Several treatments were found lethal to the larvae and eggs in cysts of the soybean nematode. When cyst material was steeped in hot water at 54–55°C for 15 minutes or at 48–49°C for 30 minutes, no new females were recovered from inoculated soybean plants, and no viable larvae were recovered in Syracuse watch glasses; however, treatment at 48–49° for 15 minutes was not effective. Steep treatments with 0.5 per cent formalin for 3 hours at 45° was also effective, but treatment with this material for as long as 4 hours at room temperature was ineffective. Soak treatments with sodium 2,4,5-trichlorophenoxide at a concentration of 3 lb. of an 85 per cent formulation to 100 gal. of water for 15 minutes and at 2 lb. to 100 gal. water for 1 hour were also lethal to the eggs and larvae in cysts. Treatment with a concentration of 2 lb. to 100 gal. of water for 45 minutes reduced viability; however, 1–6 new females were recovered from inoculated soybeans in each of the 3 tests. A comparison of the effectiveness of these materials as eradicant treatments is given in Table 1.

In the initial tests, 11 new females were recovered from 1 pot that had received cysts treated with hot water at 48–49°C for 30 minutes; 3 new females were recovered from 1 pot that received inoculum treated with 0.5 per cent formalin at 45° for 3 hours; and 2

TABLE 1.—*Recovery of new nematodes from cysts of Heterodera glycines given various steep or soak treatments and then placed in Syracuse watch glasses for up to 4 weeks, and recovery from soybeans grown in soil containing similarly treated cysts*

Material	Treatment		Average recovery[a] per test			
	Temperature °C	Duration of treatment (minutes)	From Syracuse dishes (second-stage larvae)	From soybean plants		
				Females	Males	Larvae
Hot water	48°-49°	15	++	+++	++	+
		30-60	0	0	0	0
	54°-55°	15-30	0	0	0	0
0.5% Formalin	45°	180-240	0	0	0	0
	25°-30°	180-240	++	+++	+++	+++
Dowicide B						
2 lb./100 gal.	25°-30°	20	++	+++	++	++
		30	+	++	+	+
		45	0	+	0	0
		60	0	0	0	0
3 or 6 lb./100 gal.	25°-30°	15	0	0	0	0
Water + detergent[b]	25°-30°	240	+++	++++	+++	+++
Water	25°-30°	240	+++	++++	+++	+++

[a] 0, None; +, less than 10; ++, 10–100; +++, 100–1000; ++++, over 1000.

[b] DuPont spreader-sticker (Principal functioning agents—sodium sulfates of mixed esters of long-chain alcohols and fatty acids and diethylene glycol abietate).

new females were recovered from a pot that received inoculum treated with 2 lb. Dowicide B to 100 gal. water for 60 minutes. Since these pots were adjacent to check plants and are believed to have been contaminated, these exceptions were not included in the data given in Table 1. In the remaining 3 pots in this test and in 2 subsequent tests, no reproduction was observed when cyst material received these treatments.

In addition to the intervals listed in Table 1, the Dowicide B treatment of 3 lb. to 100 gal. water was applied for 30, 60 and 120 minutes; that of 6 lb. to 100 gal. water for 30 and 60 minutes, and that of 2 lb. to 100 gal. water for 120 minutes. No nematode reproduction or evidence of viability was observed when these treatments were used. Mersolite W treatments of 1 lb. to 400 or 600 gal. water for 5 and 15 minutes, as well as New Improved Ceresan treatments of 2 lb. to 100 gal. water for 2 and 15 minutes, were ineffective. In addition, a mixture of Mersolite W, 1 lb. to 400 gal. water, and Dowicide B, 3 lb. to 100 gal. water, was tested. When treatments were applied for as long as 30 minutes, 1 to 5 new females were consistently obtained from each pot of inoculated soybeans.

None of the treaments used appeared to be injurious, with the exception of Dowicide B at 6 lb. to 100 gal. water when bulbs were soaked for 30 minutes. In this case, the bulbs and corms were injured only superficially. No differences in rate of emergence and no phytotoxicity effects were observed on either the foliage or flowers with this or any of the other treatments used.

Discussion.—The hot-water-formalin treatments of 45°C for 3 and 4 hours, which are commonly used for eradication of root-knot and bulb and stem nematodes from bulbs, bulblets, corms and cormels (2, 3), as well as the hot-water treatments of 120°F, which Feldmesser (1) reported as lethal to larvae in cysts of the golden nematode of potato, were effective as eradicant treatments for the soybean cyst nematode. Feldmesser reported that treatment with 0.5 per cent formalin at 85°F for 10 minutes was lethal to larvae in cysts of the golden nematode; however, larvae and unhatched eggs in cysts of *H. glycines* did not appear to be eradicated completely with formalin treatments at 25–30°C for 4 hours.

Tests conducted at the North Carolina Experiment Station (2) have shown that an 85 per cent sodium formulation of trichlorophenoxide, when used as a prestorage dip treatment at a rate of 2 lb. to 100 gal. water for 20–30 minutes controlled Fusarium storage rot of gladiolus. Preliminary tests on narcissus bulbs also indicate that this material may be effective in the control of Fusarium rots of narcissus when used at a rate of 3 lb. to 100 gal. water for 15 to 30 minutes (personal communication by F. A. Haasis). Hence, prestorage treatments with Dowicide B at rates of 3 lb. to 100 gal. water for 15 minutes would not only eradicate cysts of *H. glycines* contaminating bulbs or corms but might also aid in the control of Fusarium bulb and corm storage rots. A 15-minute soak treatment with this material at 3 lb. to 100 gal. water for all bulbs or corms of these 2 crops harvested from infested soil or moved outside the infested area has been recommended (6).

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FIELD AND LABORATORY TESTS OF SEED PROTECTANTS FOR CANNING PEAS[1]

D. J. Hagedorn[2]

SUMMARY

Tests in greenhouse temperature tanks, in a walk-in cold chamber, and in rolled towels were compared with field tests and were found to be useful for determining the efficacy of seed protectants for canning peas in Wisconsin. Towel and cold-chamber tests appeared to be more precise than the tank tests. KF467 and the various Phygon preparations were generally the most effective from 1949 through 1952. Orthocide 75 was superior in 1953.

The use of seed protectants for canning peas (*Pisum sativum* L.), especially the wrinkle-seeded types, is widespread and well established. New fungicides are continually being developed, however, and there is a need for rapid and accurate techniques for testing such materials. This paper is to report the results of experiments on pea seed protectants conducted in Wisconsin in the field, greenhouse, walk-in cold chamber, and laboratory from 1949 through 1953. These studies were made not only to compare the effectiveness of available pea seed protectants against damping-off primarily caused by *Pythium* spp. but also to compare the usefulness of various testing techniques.

METHODS AND MATERIALS.—The seed protectants studied were Arasan (50 per cent thiram = bis (dimethylthiocarbamoyl) disulfide), Arasan SF and Arasan SF-X (75 per cent thiram), Phygon (50 per cent dichlone = 2,3-dichloro-1,4-napthoquinone), Phygon XL (50 per cent dichlone), Spergon (96 per cent chloranil = tetrachloro-*p*-benzoquinone), Spergon SL (95 per cent chloranil), KF467 (10 per cent biethyl mercuric perthiocyanate), Dow 9B (32 per cent zinc 2,4,5-trichlorophenoxide), Vancide 51 (30 per cent sodium dimethyldithiocarbamate and sodium derivative of 2-mercaptobenzothiazole), and Orthocide 75 (75 per cent captan = *N*-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide). Vancide 51 was the only liquid fungicide studied; the rest were wettable powders or dust formulations. KF467 was an experimental mercury dust compound supplied by the Delaware Agricultural Experiment Station.

The dosages used changed with advance in time and in almost all cases were based upon current recommendations of the manufacturers. Dosages were Arasan, 2 oz./100 lb. seed in all experiments; Arasan SF, 1 lb./gal. (slurry concentration = 1 lb. seed protectant/gal. water; with size 23-ml slurry cup and seed gate No. 15, this mixture would treat 912 lb. of pea seed) in 1949 and 1950, 1½ lb./gal. in 1951 and 1952, and 2 lb./gal. in 1953; Dow 9B, 3 oz./100 lb. in 1949 and 1950, 3⅔ oz./100 lb. in 1951 and 1952; KF467, 1⅔ oz./100 lb. in all experiments; Phygon, 1 oz./100 lb. in 1949 and 1950, 2 oz./100 lb. in all other years; Phygon SP, 1 lb./gal. in 1949 and 1950, 2 lb./gal. in 1951 and 1952; Spergon, 2⅔ oz./100 lb. in 1949 and 1950, 3 oz./100 lb. in all other years; Spergon SL, 2 lb./gal. in 1951 and 1952; Vancide 51, 227 ml of 50 per cent solution/100 lb. in 1953; and Orthocide 75, 2 oz./100 lb., and 2 lb./gal. in 1953.

The slurry applications of Arasan SF, Phygon SP, and Spergon SL were made with a commercial-type treating machine; lots of 10 lb. of seed were used to provide adequate volume. Protectants used in the powder form were applied in a rotating barrel. Liquid application of Vancide 51 was made by pouring the proper amount of seed protectant (in water) over the appropriate weight of seed contained in a rotating vessel. These seeds were thoroughly mixed and spread to dry.

The 1949 studies consisted of 1) 2 field trials in prairie soil, 2) 3 temperature-tank trials in the greenhouse–1 with each of the prairie, silt loam, and muck soils; and 3) 1 trial in the walk-in cold chamber with silt loam soil. In 1950, the field tests consisted of 1 trial in prairie soil and one in silt loam. Six experiments were conducted in the greenhouse and also in the walk-in cold chamber during 1950. These consisted of duplicate trials with each of the 3 soil types mentioned above. The 2 field trials in 1951 were made in prairie and silt loam soils, and the 3 temperature-tank tests were made in prairie, muck, and compost soils. Only field studies were made in 1952; 1 trial was made in prairie soil and 1 in silt loam. These same soils were used also for the field trials in 1953. Muck soil was used for the 2 tests in the temperature tanks and for the rolled-towel tests.

The Wisconsin Perfection pea variety was used in all except the 1953 experiments, seed of the same lot number being treated in 1949 and 1951. The variety Surprise was used in the 1953 tests. The field tests consisted of 100-seed randomized plots replicated at least 4 and sometimes 6 times per trial. Most greenhouse temperature-tank tests included 6 replications of 12 seeds each for each treatment. Fifty seeds in each of 4 replications were used in the walk-in cold chamber and towel tests.

Germination percentages were recorded when the plants were 4–6 in. high in all except the towel tests. Ratings for the towel test, which was a modification of the rolled towel cold test for corn, were made after

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a week's incubation at 16°C. Normal plants were given a rating of 1, and obviously stunted plants were rated 0.5. Soil in metal pans in the greenhouse temperature tanks was held at about 12°C, and the temperature of soil in the walk-in cold chamber was 10°C. After 10 days of cold-chamber exposure, the pans containing slowly germinating seeds were removed and the seedlings allowed to develop at room temperature (about 22°C) for 10 days before data were recorded.

In the statistical analysis, the ranges of each fungicide for each trial were plotted against the mean to determine if transformations were necessary. Correlation coefficients were calculated between duplicate trials with the same soil type, with different soil types within an experiment, with different soil type and experimental combinations within a year, and with different experiments over all soil types and similar years. For the analyses of variance, data for 1949 and 1950, and also those for 1951 and 1952, were combined. The means of the replications for each fungicide for each trial were used as the experimental unit. The fungicide × soil type interaction was used as an estimate of the error variance for the calculation of LSD values. Variation within replication was also calculated for each trial.

EXPERIMENTAL RESULTS.—*Experiments in 1949 and 1950.*—Significant differences were found between treatment means in all the experiments (Table 1). KF467 and/or Phygon were the most effective, followed by Spergon, Arasan, and Phygon XL. In general, results with Arasan SF, and Dow 9B were only slightly different from those with the control.

Statistically significant correlations were observed between duplicate trials for both the 1949 and 1950 field experiments, for the 1950 greenhouse temperature-tank studies, and for the 1950 cold-chamber experiments except those involving marsh soil. Correlation coefficients between the data for studies in field and greenhouse, field and cold chamber, and greenhouse and cold chamber were statistically significant; they were greater than 0.800 in 1949 and greater than 0.774 in 1950. Correlations between the tests conducted in these 3 places, in the combined 1949 and 1950 data, were all statistically significant, the coefficients of correlation were greater than 0.920. The data indicate that either the greenhouse or cold-chamber tests could be used to determine the efficacy of a fungicide under field conditions.

TABLE 1.—*Combined mean percentage germination, expressed in angles[a] of pea seeds treated with various protectants, and ranks[b] of the protectants tested during 1949 and 1950 in field, greenhouse, and cold chamber*

Seed protectant	Field		Greenhouse		Cold chamber	
	x	Rank	x	Rank	x	Rank
Arasan	78.2	3	52.1	4	29.4	4
Arasan SF	64		35.2		22	6
Phygon	86.7	1	62	2	32.2	2
Phygon XL	66.5	5	40.1	5	22.8	5
Spergon	77	4	53.1	3	31.7	3
KF467	82.2	2	67.3	1	35.4	1
Dow 9B	64.5	6	25.2		13.6	
None	56		29.1		11.1	
LSD (0.05)	8.2		10.3		7.2	
(0.01)	11.2		13.9		9.8	

[a] Angle = arc sin $\sqrt{\text{percentage}}$.
[b] Only those means that were significantly different from the control were ranked.

TABLE 2.—*Combined mean percentage germination of pea seeds treated with various protectants, and rank[a] of the protectants tested during 1951 and 1952 in field and greenhouse tests*

Seed protectant[b]	Field		Greenhouse	
	Mean percentage germination	Rank of protectant	Mean percentage germination	Rank of protectant
Arasan	53.5	8	61.7	4
Arasan SF (1×)	56.5	5.5	43.3	
Arasan SF (2×)	58.7	4	51	9
Phygon	56.5	5.5	60.7	6
Phygon SP (1×)	60.2	3	66	2
Phygon SP (2×)	62	2	67.3	1
Spergon	52	10	58	7
Spergon SL (1×)	53.2	9	54.3	8
Spergon SL (2×)	54.2	7	61.3	5
KF467	63	1	65.7	3
Dow 9B	44		44.7	
None	40.7		34.7	
LSD (0.05)	7.4		14.5	
(0.01)	9.9		19.6	

[a] Only those means that were significantly different from the control were ranked.
[b] 1× = recommended dosage; 2× = double recommended dosage.

Experiments in 1951 and 1952.—The analyses of variance of the 1951 and 1952 data showed significant differences between seed protectants in both field and greenhouse experiments (Table 2). KF467 and Phygon were generally the most effective. The Spergon and Arasan compounds were less beneficial than the above, and Dow 9B was little different from the untreated control.

Statistically significant correlations were found between duplicate trials in both the 1951 and 1952 field experiments. Such correlations were also obtained between the 1951 field experiments and greenhouse experiments with prairie and compost soil. Results with these 2 soils in the greenhouse were also significantly correlated. There was no correlation between the greenhouse experiments with muck soil and other greenhouse or field experiments. A coefficient of correlation of 0.448, which was not statistically significant, was obtained between the field and greenhouse experiments in the combined 1951 and 1952 data. Since the 1951 data (with the 1 exception mentioned above) showed a significant correlation between the experiments, and there was some indication of a correlation in the 1951-1952 combined data, it again appeared that greenhouse tests could be used to predict field response to pea seed protectants.

TABLE 3.—*Combined mean percentage germination, expressed in angles[a] of pea seeds treated with various protectants, and rank[b] of the protectants tested during 1953 in the field, greenhouse, and paper towels*

Seed protectant	Field		Greenhouse		Towels	
	x	Rank	x	Rank	x	Rank
Arasan SF-X	75.5		39.5	6	61.5	6
Orthocide 75	85	1	87.5	1	89	1
Orthocide 75 (slurry)	77.5	4	74.5	3	80.5	4
Phygon	78	3	83.5	2	88	2
Spergon	82.5	2	69	4	81	3
Vancide	77	5	60.5	5	72.5	5
None	62.5		15.5		21	
LSD (0.05)	14.4		7.3		19.3	
(0.01)	21.8		11.0		29.3	

[a] Angle = arc sin $\sqrt{\text{percentage}}$

[b] Only those means that were significantly different from the control were ranked.

Experiments in 1953.—Significant differences were found between treatment means in all experiments (Table 3). Orthocide 75 was the most effective seed protectant, followed in order by Phygon and/or Spergon, Orthocide slurry, Vancide 51, and Arasan SF.

Significant correlations were obtained between duplicate trials in the greenhouse and towel experiments but not in the field studies. Field results from the first loam soil trial were highly correlated with those of both greenhouse and towel experiments, but there was no correlation between the data obtained in the second field trial and those obtained in either of the other tests. When the data were combined, correlations between the field and towel experiments and those between the towel and greenhouse experiments were highly significant ($r > 0.938$). The field and greenhouse correlation ($r = 0.721$) just approached statistical significance.

DISCUSSION.—Hoppe[3], working with corn seed, was one of the first to establish the value of modern laboratory tests for the evaluation of seed protectants. He showed that tests made in pans placed in a walk-in cold chamber or tests made in glass tumblers or in paper towels containing infested soil and placed in a refrigerator, yielded results that were highly correlated with those obtained in the field. The writer's studies show that pea seed protectants also can be evaluated by laboratory tests.

The results of these studies were as consistent as could be expected, considering the diverse nature of the tests and the fact that they were conducted over a period of 5 years and on several soil types. In the 1949 and 1950 experiments, the variation within replicates in the greenhouse was quite large, compared to that in the field tests and that in cold-chamber tests, suggesting that the cold-chamber test was more precise than the greenhouse method. The high error variance in the greenhouse experiments might have been reduced if the number of seeds per sample had been increased. The 1951-1952 results were not as conclusive as the earlier data. In the 1953 experiments, the variation within replications was considerably smaller for the towel tests than for the greenhouse experiments. The data indicated that either the greenhouse or towel test could be used to test the efficacy of a seed protectant for peas; however, for precision, the towel test seemed more satisfactory.

There were statistically significant differences among replications in the field tests in all 5 years, suggesting that the randomized complete block design used was very satisfactory. Although many of the greenhouse, cold-chamber, and towel tests gave significant differences among replications, some others failed to do so. In the "laboratory" tests, then, a completely randomized design would apparently be a more efficient design than a randomized complete block design.

In most tests, dry application of a seed protectant was superior to use of the slurry seed-treating method. Although a few tests indicated that doubling the amount of seed protectant was beneficial, this procedure generally did not appreciably increase germination.

The 3 seed protectants KF467, Phygon, and Orthocide gave the best and most consistent results. Only the last 2 fungicides are now available for commercial use. It is significant to note that Spergon, which at present is the seed protectant most widely used commercially for canning peas, did not rank high consistently and in some tests gave very mediocre results. These results are similar to those recently reported by de Zeeuw and Guyer[4] in Michigan.

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[4] de Zeeuw, D. J., A. L. Andersen, and G. E. Guyer. 1956. Comparison of fungicide and fungicide-insecticide seed treatments of peas and beans. (Abs.) Phytopathology 46:10.

PATHOLOGICAL HISTOLOGY AND VARIETAL REACTIONS IN SEPTORIA LEAF BLOTCH OF BARLEY [1]

G. J. Green[2] and J. G. Dickson[3]

SUMMARY

Plant disease survey records indicate that Septoria leaf blotch (incited by *Septoria passerinii* Sacc.) recently has become a more important factor in barley production than it has been in the past. The disease developed most rapidly between 20° and 24°C. Spraying plants in the field or greenhouse with a water suspension of spores containing 0.5 per cent gelatin was a reliable method of obtaining infection. The *S. passerinii* occurring widely on *Hordeum jubatum* L. in Manitoba was found to be pathogenically distinct from that attacking cultivated barley. It was concluded that *H. jubatum* was not a factor in the epidemiology of the disease on barley.

Penetration by *S. passerinii* was found to be stomatal in resistant and susceptible varieties. In leaves of susceptible varieties, the sparse intercellular hyphae without haustoria incited necrosis of the mesophyll cells but did not commonly attack other tissues of the leaves. In resistant varieties, the mycelium was much restricted. Host reaction to the fungus was manifested by a thickening of the cell walls in contact with hyphae. This reaction, more pronounced in the most resistant varieties, suggested that the response in the cell wall may contribute to resistance.

Varietal tests in the field and greenhouse with 195 barley varieties revealed that at least 53 varieties, mostly of the Coast or North African type, were resistant to the disease. All varieties of the Manchurian type tested were susceptible.

Septoria leaf blotch (incited by *Septoria passerinii* Sacc.) has not been investigated experimentally since 1923, when Weber[4] described its symptoms and reported briefly on the morphology and pathogenicity of the fungus in a paper dealing with several species of *Septoria*. The lack of interest in the disease indicated that it was regarded as an unimportant factor in barley production; however, the more recent prevalence of the disease on the important malting barley varieties has stimulated renewed interest in the disease and its control. In 1943 and succeeding years, Johnson[5] reported heavily infected fields in Manitoba. Atkinson[6] reported in 1944 that the disease was widespread throughout North and South Carolina but that "as yet it is of no economic importance." Chester[7] reported in 1948 that "in Oklahoma barley was more heavily attacked by this disease than at any past time in the memory of many cerealists." From 1949 to 1952 inclusive, the senior author examined 161 barley fields in Manitoba. In 1949, the epiphytotic was light: 16 fields of 21 examined showed a trace of the disease and the others were free from it. The epiphytotic was also light in 1951: of 42 fields examined only 5 had heavy or moderate infections, 4 had light infections, 19 had a trace of the disease, and 14 were free from it. Infections were much heavier in 1950 and 1952. Ninety-eight fields were examined in these years; 35 of them had heavy or moderate infections, 26 had light infections, 27 had a trace of the disease, and 10 were free from it.

In 1953 and 1954 the junior author found barley severely damaged by the disease in local areas in North and South Dakota, Minnesota, and Wisconsin. In 1955, Septoria blight was present in epidemic form throughout the north-central area of the United States, especially on the variety Kindred. The disease appeared to be a major factor in causing low yields and light kernels.

These reports from several barley-producing areas in North America indicated an increase in the prevalence and importance of Septoria leaf blotch, and prompted this study with a view to discovering sources of resistance and investigating the pathological histology of the disease in susceptible and resistant varieties.

MATERIALS AND METHODS.—Host penetration and pathological histology were investigated on plants in the 3- or 4-leaf stage grown in the greenhouse. Host penetration was studied mostly on the susceptible varieties Montcalm and Vantage and the resistant varieties Atlas and Feebar. Microscopic observations were made on 1-cm-square portions of inoculated leaves that had been cleared in a solution of glacial acetic acid and absolute alcohol and mounted in lactophenol and cotton blue. These observations were confirmed by examination of paraffin sections of inoculated leaves. The pathological histology of the disease was studied in

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[4] Weber, G. F. 1923. III. Septoria diseases of rye, barley and certain grasses. Phytopathology 13: 1-23.

[5] T. Johnson in the Twenty-third (1943), Twenty-fifth (1945), Twenty-sixth (1946) and Twenty-seventh (1947) Annual Reports of the Canadian Plant Disease Survey, edited by I. L. Conners and published by the Canada Department of Agriculture, Science Service, Botany and Plant Pathology Division, Ottawa, Ontario.

[6] Atkinson, R. E. 1944. Cereal diseases in the Carolinas. U. S. Dept. Agr. Pl. Dis. Rptr. 28: 288-289.

[7] Chester, K. S. 1948. Outbreak of wheat covered smut, and other unusual developments of cereal diseases in Oklahoma. U. S. Dept. Agr. Pl. Dis. Rptr. 32: 320-321.

leaf sections prepared by the paraffin method. The leaves were killed and fixed in formalin:acetic acid: alcohol solution, dehydrated with tertiary butyl alcohol, imbedded in paraffin, cut with a rotary microtome, and stained with Conant's quadruple combination. The diseased leaf tissues were collected daily, beginning 24 hours after inoculation, from plants of the varieties Vantage, Montcalm, Atlas, and Feebar, and from Wisconsin H-106-1, which was indeterminate in reaction.

In the greenhouse, plants were inoculated by spraying them with water suspensions of conidia or pycnidiospores. The inoculum usually contained 0.5 per cent gelatin to ensure heavy infection and was applied with an atomizer. The inoculated plants were incubated for 48 hours in a very moist atmosphere at about 20°C.

Field inoculations were carried out by a method similar to that used in the greenhouse. The inoculum was applied by means of a 2½-gal. portable pressure sprayer just before the plants headed. Replicated tests demonstrated that the addition of gelatin to the inoculum was especially important in the field. In 1951, conditions at Winnipeg did not favor infection, and plants sprayed with inoculum lacking gelatin were lightly infected whereas plants sprayed with inoculum containing 0.5 per cent gelatin were heavily infected. In 1952, conditions were more favorable for infection and inoculum lacking gelatin produced a fairly heavy infection, but the amount of infection was increased where gelatin was added. The addition of gelatin to the inoculum increased the effectiveness and reliability of field inoculations with this fungus and made possible fairly extensive field trials.

Varietal reactions to the disease were determined by seedling tests in the greenhouse and by adult-plant tests in the field. Several cultures of *S. passerinii* isolated from barley grown in southern Manitoba were used to produce inoculum for the tests. Seedling reactions were recorded 12–16 days after inoculation. Field reactions were determined about a month after inoculation. The resistance or susceptibility of each variety was assessed by estimating the percentage of the leaf tissue killed. The appearance of the different types of reaction is discussed under the description of the disease.

DESCRIPTION OF THE DISEASE.—*S. passerinii* may attack the leaf blades, leaf sheaths, floral bracts, and awns of barley plants. In the field, isolated lesions appear on young plants early in the season. These grayish-green or straw-colored very elongate lesions soon contain many dark brown pycnidia. Lesions on older plants are usually straw-colored and coalesce to form indefinite, irregular, dead blotches on the leaf blades (Fig. 1, B). On the leaf sheaths the disease causes a gray discoloration of the affected tissue that is conspicuous on mature plants (Fig. 1, C). The most reliable diagnostic feature of the disease is the abundant pycnidia that appear in the dead areas. Pycnidia often develop on the awns of heavily infected plants but usually do not appear on the kernel.

Symptoms on artificially inoculated seedlings of susceptible varieties in the greenhouse (Fig. 1, A) are somewhat different from symptoms in the field. Elongate, indefinite, chlorotic areas appear on infected leaves 9 or 10 days after inoculation under conditions favorable to the disease. About 3 days later, the chlorotic areas develop into grayish-green or straw-colored sunken lesions with many pycnidia. Lesions vary from 5 to 7 mm in length and from 1½ to 3 mm in width. Leaves may be so heavily infected that lesions coalesce, killing the distal third or more of the leaf. On seedlings of varieties intermediate in reaction, the disease causes relatively small, more sharply defined chlorotic areas that develop into straw-colored lesions in which pycnidial production is often delayed and sparse. On seedlings of resistant varieties, symptoms vary from straw-colored necrotic flecks to small chlorotic areas that develop into small straw-colored lesions without pycnidia. Some barley varieties are so variable in symptom expression that the disease reaction was classified as indeterminate.

PATHOGEN.—Only the imperfect stage (*S. passerinii*) of the leaf blotch pathogen is known. The fungus produces a sparse mycelium in the leaf blades and sheaths of barley plants; later ostiolate pcynidia develop under the stoma. Pycnidiospores are exuded in cirri in a gelatinous, water-soluble matrix that permits spore dissemination by wind and water. Pycnidia from 7 collections of the fungus averaged 100.4μ in diameter. The average size of pycnidiospores from 10 collections was 36.1 × 2.4μ. Occasionally, microspores were observed exuding from pycnidia with macrospores. Microspores observed in 4 collections averaged 9.8 × 1.3μ. These dimensions of pycnidia and macrospores agree with those given by Sprague[8], but the dimensions of the microspores are considerably larger than those recorded by Sprague.

Viable pycnidiospores were present in pycnidia on crop remains in April at Madison, Wisconsin[4]. Microscopic examination showed that only 1 of 29 pycnidia from overwintered crop residue collected on May 24 at Winnipeg contained a small number of spores. After 4 days in a moist chamber at room temperature, 7 out of 8 pycnidia examined contained macrospores, and 5 of these also contained microspores. Macrospores and microspores were found on June 2 and 10 in pycnidia from overwintered straw. These spores infected barley seedlings in greenhouse tests. It seems likely that at Winnipeg this fungus overwinters largely as mycelium and produces new spores in the spring.

Septoria passerinii grows readily on potato-dextrose agar. Pycnidiospores, which exude from pycnidia placed in water, germinate by budding-off conidia which in turn bud-off other conidia to form pink, mucous colonies with very little mycelial development.

[8] Sprague, R. 1950. Diseases of cereals and grasses in North America. Ronald Press, New York. 538 p.

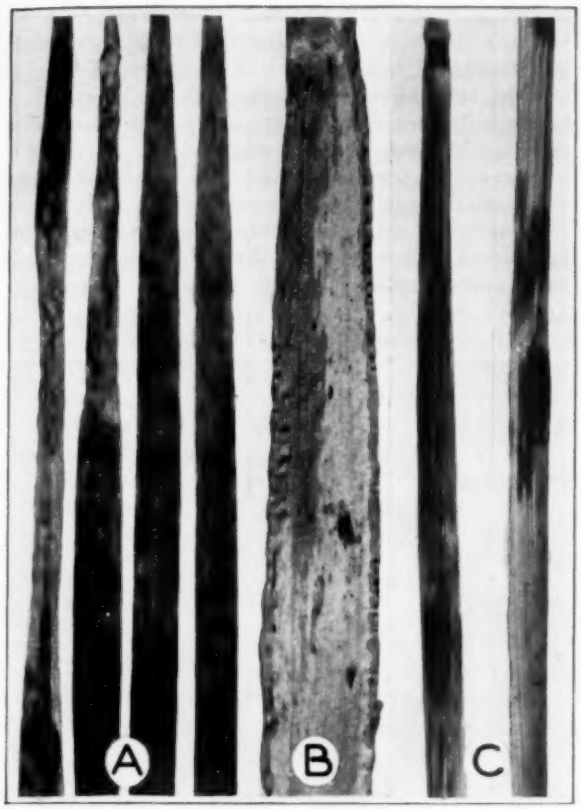

FIG. 1. Symptoms of Septoria leaf blotch on A) seedling leaves of Montcalm barley, grown in the greenhouse, 14 days after inoculation with *Septoria passerinii*; B) leaf of an adult plant from the field; and C) leaf sheaths of mature plants from the field.

In about 6 days, the colonies on media either turn black and hard and produce a small amount of mycelium or they remain pink and become quite firm. For infection studies, it is best to wash the conidia from the agar while the colonies are still mucous. Pycnidia have not been observed in culture.

The temperature response of the fungus is similar in the host and on artificial media. Tests at the University of Wisconsin, Madison, showed that the disease developed most rapidly and typically at temperatures from 20° to 24°C. Disease development is slightly faster at the higher temperature, but at 20°C the plants are more thrifty. The percentage germination of conidia on artificial media after 20 hours incubation at different temperatures was 3 per cent at 12°C; 10 per cent at 16°; 65 per cent at 20°; 72 per cent at 24°; 38 per cent at 28°; and 22 per cent at 32°.

S. passerinii, which according to Sprague[8] occurs in Iowa, Minnesota, North Dakota, South Dakota, and Wisconsin on *Hordeum jubatum* L., also occurs widely in Manitoba on this species. To determine whether the fungus that occurs on *H. jubatum* is pathogenically the same as that occurring on barley, 16 varieties of barley (Feebar, Atlas, Moore, Vantage, Montcalm, Peatland, Algerian, Nepal, Carre 180, Cebada Capa, Morocco, Kindred, Wis. H-106-1, Dorsett, Afghan II, and Valentine), Marquis wheat, Rosen rye, and Victory oats were inoculated with 6 cultures of *S. passerinii* from *H. jubatum* collected in Manitoba at St. Norbert, Manitou, Portage la Prairie, Deloraine, and Starbuck (2 cultures). Three cultures of *S. passerinii* isolated from cultivated barley from Souris, Manitou, and Benito in Manitoba and 2 cultures from *H. jubatum* from Starbuck and Deloraine in Manitoba were tested for pathogenicity on *H. jubatum*.

None of the isolates from *H. jubatum* attacked the varieties of cultivated barley, wheat, rye, or oats. *H. jubatum* was resistant to the isolates from cultivated barley, but it was susceptible to the isolates from *H. jubatum*. Weber[4] also was unable to obtain infection of *H. jubatum* with *S. passerinii* isolated from *H. vulgare*. Evidently the species of *Septoria* attacking wild barley in Manitoba, in part at least, is pathogenically distinct from that attacking cultivated barley. The resistance of *H. jubatum* to *S. passerinii* from cultivated barley indicates that this plant species is of little importance in the epidemiology of Septoria leaf blotch.

PATHOLOGICAL HISTOLOGY OF SEPTORIA LEAF BLOTCH IN RESISTANT AND SUSCEPTIBLE BARLEY VARIETIES.—On barley leaves, the germination of conidia and of pycnidiospores was similar, although 2 differences were observed. Usually germ tubes grew from one or both ends of conidia and pycnidiospores, but occasionally a germ tube of relatively small initial diameter grew from the side of a pycnidiospore. Also, conidia frequently produced small aseptate or uniseptate secondary conidia at their septa, whereas pycnidiospores produced these secondary spores rarely. Such differences apparently had little effect on the manner of host penetration or disease development.

Germ tubes, which sometimes branched, grew indiscriminately over the leaf surface or along the depressions over adjoining walls of epidermal cells. Elongation along these depressions sometimes was initiated by a swelling that suggested the initial stage of appressorium formation, but the germ tubes developed normally beyond the swellings. Because direct penetration had been reported previously[4], special attention was given these swellings. When leaves of Vantage barley were inoculated with a suspension of pcynidiospores, germ tubes up to twice the length of the spores were observed 24 hours later but no penetrations were found. Forty-eight hours after inoculation, the germ tubes were many times the length of the spores. In a few instances, the tip of a germ tube appeared to have penetrated the cuticle over the adjoining walls of epidermal cells, but it had not penetrated between the cells. In several other instances, swellings in the germ tubes suggested that an unsuccessful attempt at direct penetration had been followed by continued elongation. Several germ tubes were over stomata, but they had not entered

the leaf. Seventy-two hours after inoculation, penetration through the stomata and mycelial development in the leaf were common. When a germ tube contacted a stoma, it frequently thickened and passed between the guard cells, branching as it entered the leaf (Fig. 2, A, C). Hyphae were observed on the inner surfaces of the guard and accessory cells and in the substomatal cavity. Similar observations were made many times on leaves inoculated with conidia. Evidence of penetration between the epidermal cells was observed twice. The importance of such a rare event was obscure, but it might be an indication of an alternative mode of penetration which might operate under different conditions.

Penetration of the resistant varieties Atlas and Feebar was identical with penetration of the susceptible varieties Montcalm and Vantage.

Disease symptoms on young plants of the susceptible varieties Vantage and Montcalm consisted of pale chlorotic areas which developed into grayish-green or tan-colored sunken lesions. Although lesions on Montcalm were sometimes smaller and more sharply de-

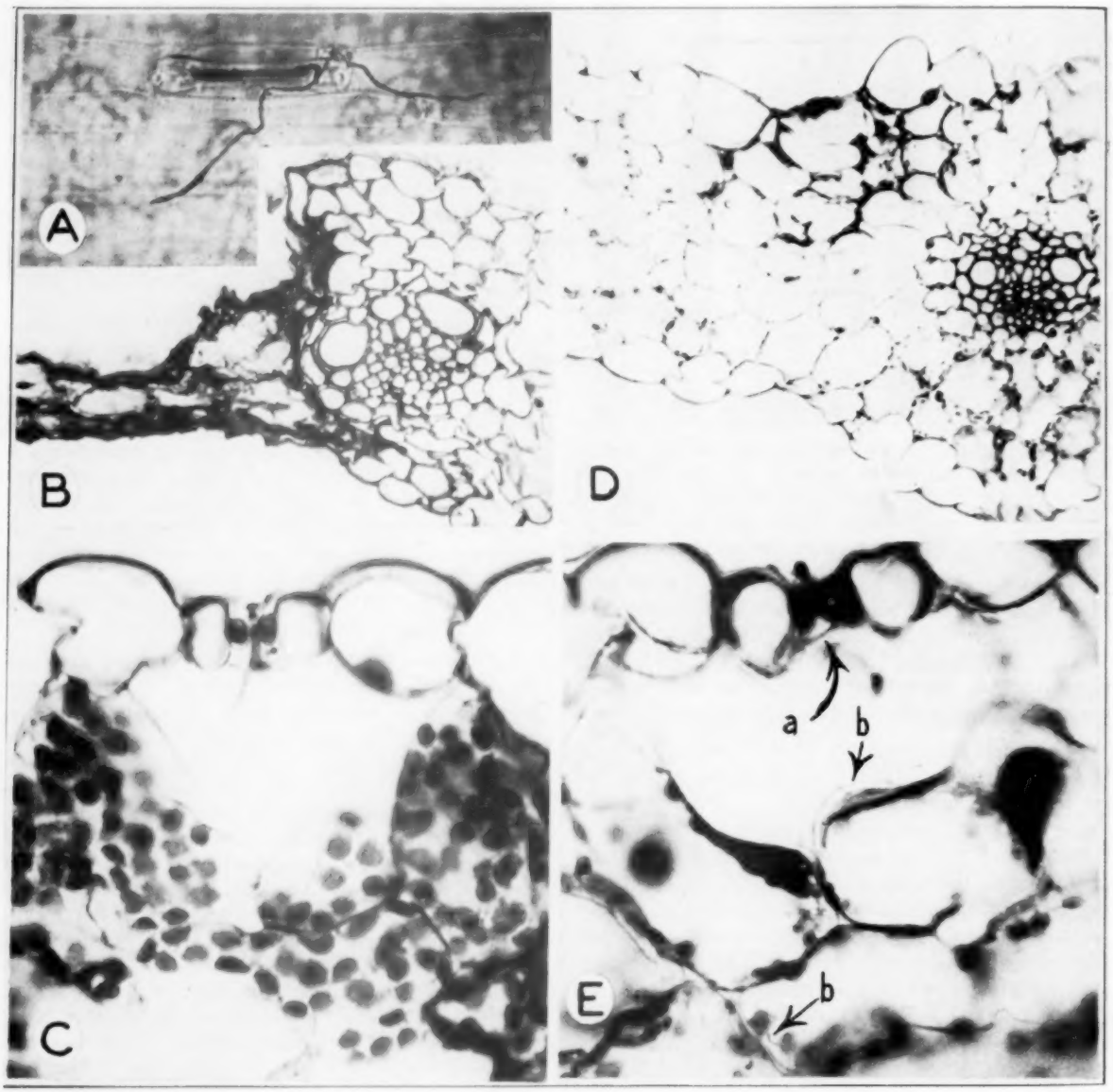

FIG. 2. A) Stomatal penetration by *S. passerinii* on a leaf of Moore barley (540×). B) Septoria leaf blotch lesion limited laterally by a vascular bundle (150×). C) Stomatal penetration into a leaf of Atlas barley by *S. passerinii* (1125×). D) Moderately resistant reaction of the barley variety Wisconsin H-106-1 (260×). E) Hyphae of *S. passerinii* in a leaf of the resistant barley variety Atlas. a) Hyphae under damaged guard cells of a stoma through which penetration occurred. b) Hyphae next to thickened host cell walls (1125×).

fined than those on Vantage, leaf sections revealed no differences in the reaction of the 2 varieties. After penetration, hyphae grew most rapidly directly beneath the epidermis, usually running from stoma to stoma, but never penetrating between the epidermal cells. Other intercellular hyphae grew in the mesophyll tissue but did not produce haustoria. As the sparse mycelium reached its maximum development, pycnidia developed under the stomata, usually under those on the upper surface of the leaves. The lateral advance of the hyphae in older tissue was prevented by the larger vascular bundles (Fig. 2, B), and lesions were elongate. In young tissue the bundles and their sheaths were not as fully developed, and the mycelium penetrated between sheath and epidermis to produce an oval lesion. Mesophyll tissue was the first to show necrosis. Epidermal cells usually were not affected until the underlying mesophyll had been destroyed. Occasionally outer cells of the vascular bundle sheaths were damaged, but hyphae rarely advanced between them. Hyphae were never found in or among the xylem and phloem elements.

Chloroplasts, cytoplasm, and cell walls stained less intensely with fast green in leaf sections showing early infections than in healthy tissue. All cells in an infected area showed similar effects, although, as far as could be determined, they were not all in contact with hyphae. As the disease progressed, the reticulate network of the nuclei of the host cells disappeared, and the nuclei became shrunken and homogenous and apparently stained with safranin instead of with crystal violet. In the late stages of lesion formation, the protoplast components were shrunken and disorganized. The cytoplasm, chloroplasts, and cell walls in the diseased tissues showed greater affinity for safranin than for fast green, which stained these structures in healthy cells. A safranin-stained substance resembling degenerated protoplasm appeared rarely in the intercellular spaces. Finally, the cells collapsed to form the sunken lesions characteristic of the disease. This complex of reactions to the fungus on the part of the host cells made it impossible to determine accurately which cellular component was affected first. The shrinkage of the cell contents without intercellular deposits suggested that water was lost by the cells as the disease progressed.

Symptoms exhibited by variety Wisconsin H-106-1 ranged from small, sharply chlorotic areas to small lesions. The reaction was classified as indeterminate because the chlorotic response indicated moderate resistance and the small lesions moderate susceptibility. Sections of the moderately susceptible type of lesion showed histological features similar to those described for Montcalm and Vantage, although the mycelium was less extensive. In sections of moderately resistant areas of leaves collected 10 days after inoculation, the cells longest in contact with the fungus (guard cells, accessory cells, and mesophyll cells bordering the substomatal cavity) had been killed (Fig. 2, D). Farther from the stomata the cells were less severely affected. Hyphae were difficult to find among the dead cells, but a small amount of mycelium was observed readily in the chlorotic region around the dead area. Many cell walls in contact with hyphae were thickened. As these thickenings appeared to be characteristic of the resistant reaction to this fungus, they will be discussed under the variety Atlas.

Symptoms on the resistant variety Atlas varied from straw-colored flecks to chlorotic areas that gradually became necrotic. Some inoculated leaves failed to exhibit macroscopic symptoms. In such leaves, and in leaves that produced only flecks, the fungus did not grow beyond a small area below the stoma. In the chlorotic areas the fungus was found deeper in the mesophyll.

The histological features of the chlorotic areas were similar to those of the moderately resistant lesions on the variety Wisconsin H-106-1 except that the cell-wall thickenings already mentioned were more frequent and pronounced (Fig. 2, E). This host reaction to the fungus was evident in the highly resistant fleck reaction, where only those cells adjacent to the stoma through which penetration occurred were affected. In the early stages of the interaction that gave rise to the thickening, the cell wall showed a safranin-stained fringe at the point of contact with the hyphae. Later the protoplast contracted at the point of contact forming a depression, and new material apparently was deposited on the cell wall in contact with the hyphae. The wall thickening, therefore, appeared to consist of the primary cell wall and substances that diffused from the protoplast. The staining reaction of the thickened walls differed from that of the normal walls, since the thickened portions stained brightly with safranin whereas normal walls stained with fast green. The mycelium generally stained with safranin, but hyphae in contact with thickened walls usually stained with fast green and sometimes these hyphae were considerably swollen. In many instances the normal safranin-stained hyphae blended into the green-stained region near the thickened host cell wall. The significance of this inversion of staining characteristics was not clear, but it indicated a complex host-parasite interaction. The walls of some resistant host cells did not thicken when contacted by hyphae. Whereas time and space relationships might explain the absence of thickening on some cells, there seemed to be differences in the capacity of different cells to respond to the fungus. A similar observation was made by Fellows[9] in his studies on *Ophiobulus graminis* Sacc.

In the variety Atlas, hyphae were not always in contact with cell-wall thickenings, but a granular material resembling degenerated hyphae was observed. Apparently the resistance of Atlas resulted from a host-parasite interaction that was harmful to local

[9] Fellows, H. 1928. Some chemical and morphological phenomena attending infection of the wheat plant by Ophiobulus graminis. Jour. Agr. Res. 37: 647-661.

areas of the host and pathogen and that restricted or often prevented growth of the fungus.

The variety Feeber is highly resistant to the disease. In the greenhouse, symptoms ranged from small, straw-colored, necrotic flecks to chlorotic areas which occurred only on leaves infected at a very early stage of development. Frequently, no macroscopic symptoms resulted from inoculation. Leaf sections of the variety showed the same histological reactions as leaf sections of Atlas; in Feebar, however, growth of the fungus was restricted even more than in Atlas and the host cell reaction was more pronounced.

Reaction of varieties.—The barley varieties were classified as susceptible, intermediate, or resistant based on their reaction to inoculation with several isolates of the pathogen in the seedling stage in the greenhouse and in field inoculations in 1951 and 1952.

Susceptible.—Varieties of *Hordeum vulgare* L. emend. Lam. found to be susceptible: Afghan II, C.I. 4156-2[10]; Afghan II, C.I. 6366; Apalan, C.I. 1347; Bark, C.I. 2793; Black Hull-less, C.I. 666; Byng, C.I. 6089; Callas, C.I. 2440; Chevron, C.I. 1111; Club Mariout, C.I. 261; Coast, C.I. 510; Cross, C.I. 1613; Cross, C.I. 2492; Cruzat, C.I. 6482; Duplex, C.I. 2433; Egyptian Sudan, C.I. 6489; Featherston, C.I. 1120; Galore, C.I. 7150; Gartons, C.I. 645; Gatami, C.I. 2276; Glabron, C.I. 4577; Grossklappige, C.I. 6485; Hero, C.I. 1286; Hero, C.I. 4602; Hooded Spring, C.I. 716; Horsford, C.I. 507; Horsford, C.I. 877; Horsford, C.I. 1775; Kindred, C.I. 6969; Korsbyg, C.I. 918; Kwan, C.I. 1016; Lico, C.I. 6279; Lion, C.I. 923; Malting, C.I. 1129; Manchuria, C.I. 2330; Manchurian, C.I. 739; Mansfield, C.I. 2241; Mensury, C.I. 4696; Minsturdy, C.I. 1556; Montcalm, C.I. 7149; Moore, C.I. 7251; Nepal, C.I. 595; Newal, C.I. 6088; Nobarb, C.I. 7148; O.A.C. 21, C.I. 1470; Oderbrucker, C.I. 940; Oderbrucker, C.I. 957; Oderbrucker, C.I. 4666; Odessa, C.I. 182; Olli, C.I. 6251; Pannier, C.I. 1330; Paso, C.I. 5047; Pearl, C.I. 4834; Peatland, C.I. 5267; Peru, C.I. 2302; Plush, C.I. 7030; Pontiac, C.I. 4849; Prospect, C.I. 6339; Recka I, C.I. 5051; Regal, C.I. 5030; Sacramento, C.I. 4108; Sahara, C.I. 3770; Silver King, C.I. 890; Star, C.I. 1701; Stella, C.I. 2678; Titan, C.I. 7055; Trebi, C.I. 936; Tregal, C.I. 6359; Vantage, C.I. 7324; Velvet, C.I. 4252; Velvon, C.I. 6109; Virginia Hooded, C.I. 2290; Warrior, C.I. 6991; Wisconsin Barbless, C.I. 5105; C.I. 3737; and C.I. 4223-2.

Varieties of *Hordeum distichum* L. emend. Lam. found to be susceptible: Alpha, C.I. 959; Archer, C.I. 1031; Bavaria, C.I. 6395; Charlottetown 80, C.I. 2732; Chevalier, C.I. 278; Clifford, C.I. 1910; Compana, C.I. 5438; Danubian, C.I. 6525; Duckbill, C.I. 1916; Frankonian, C.I. 680; French Chevalier, C.I. 175; Gold, C.I. 1145; Golden Drop, C.I. 2135; Golden Pheasant, C.I. 2488; Goldfoil, C.I. 928; Gordon, C.I. 4842; Halikonohra, C.I. 6004; Hanna, C.I. 203; Hanna, C.I. 906; Hanna, R.L. 117[11]; Hannchen, C.I. 531; Heil's Hanna, C.I. 682; Horn, C.I. 926; Italian, C.I. 914; Kuban Summer, C.I. 6480; Lechtaler, C.I. 6488; Michigan Two-Row, C.I. 2782; Persicum, C.I. 6531; Plumage Archer, C.I. 5033; Sanalta, C.I. 6087; Spartan, C.I. 5027; Steigum, C.I. 907; Svanhals, C.I. 187; Svansota, C.I. 1907; White Smyrna, C.I. 195; White Smyrna, C.I. 910; and White Smyrna, C.I. 2080.

Resistant.—Varieties of *H. vulgare* found to be resistant: Abyssinia, C.I. 2192; Algerian, C.I. 1179; Anoidium, C.I. 7269; Arequipa, C.I. 2329; Arlington Awnless, C.I. 702; Atlas, C.I. 4118; Barley 305, C.I. 6015; Batna, C.I. 3391; Beecher, C.I. 6566; Beldi Giant, C.I. 2777; Bolivia, C.I. 1257; Bonneville, C.I. 7248; California Feed, C.I. 799; California Mariout, C.I. 1455; Carre 26, C.I. 3386; Carre 180, C.I. 3390; Cebada Capa, C.I. 6193; Chilean D, C.I. 1433; Coast, C.I. 276; Dorsett, C.I. 4821; Feebar, C.I. 7260; Gatami, C.I. 575; Hey Special, C.I. 6487; Himalaya, C.I. 620; Juliaca, C.I. 1114; Luth, C.I. 972; Mecknos Moroc, C.I. 1379; Modia, C.I. 2483; Morocco, C.I. 6311; Orge 083, C.I. 4974; Orge 02, C.I. 4975; Peruvian, C.I. 935; Peruvian Sel. 19, C.I. 6568; Quinn, C.I. 1024; Rabat (Orge 079), C.I. 4979; Stavropol, C.I. 2103; Sulu, C.I. 1022; Telli, C.I. 194; Vaughn, C.I. 1367; White Gatami, C.I. 920; C.I. 2538; C.I. 2542; C.I. 4356; C.I. 5326; C.I. 5366; C.I. 6306; and 36Ab 6127, C.I. 7008.

Varieties of *H. distichum* found to be resistant: Blackhull, C.I. 878; Jet, C.I. 967; C.I. 4219; C.I. 4220-1; C.I. 5862; and C.I. 5863.

The reaction of some varieties was inconsistent, and they were classified as indeterminate. Varieties of *H. vulgare* placed in this class are Abacus, C.I. 1088; Baker, C.I. 975; Cape, C.I. 557; Chilian Brewing, C.I. 657; Coast, C.I. 690; Colsess, C.I. 2792; Egypt 4, C.I. 6481; Gem, C.I. 7243; Morocco, C.I. 3902-1; Purple Nepal, C.I. 2242; Valentine, C.I. 7242; and Wisconsin H-106-1. Varieties of *H. distichum* classified as indeterminate are Baku (C.I. 709) and Princess (C.I. 529).

Discussion.—In addition to the major intervarietal differences in the symptoms of Septoria leaf blotch, minor intravarietal differences that presumably are caused by varying environmental factors occur in different tests. There also are small differences in symptoms on the same plant because young leaves are more susceptible than mature leaves. In this report, macroscopic symptoms are described in relation to the histological features of the infection as far as possible in the hope that the description of the host-parasite relationship will assist in evaluating the reaction of varieties.

It is evident that infection of susceptible plants by *S. passerinii* results in the death of the host cells

[10] C. I. refers to the accession number of the Cereal Crops Section, U. S. Department of Agriculture.

[11] R. L. refers to the accession number of the Cereal Breeding Laboratory, Canada Department of Agriculture, Winnipeg.

without manifest inhibition of the fungus. Apparently the host cells are killed slowly by a substance or substances produced in the host-parasite interaction that do not inhibit the fungus. In contrast, the entrance of the fungus into plants of resistant varieties initiates a reaction deleterious to both host cells and fungus. The most notable result of the interaction is the thickening of the host cell walls in contact with hyphae. There appears to be a contraction of the protoplast at the point of contact between host cell and fungus and the formation of a thickened wall over the damaged area of the cell. These changes together suggest a complex defensive mechanism on the part of the host. The thickening of the cell walls, which is the first evidence of resistance seen in sections with the aid of a microscope, suggests that differences in the reaction of varieties may be related to differences in the composition and structure of their mesophyll cell walls as well as to differences in their metabolic activity.

Many commercial malting varieties of the Manchurian-Oderbrucker type, such as O.A.C. 21, Montcalm, and Kindred, as well as some high yielding feed barleys such as Vantage, Plush, and Newal, are susceptible to the disease. Several of the better known varieties of *H. distichum*, such as Compana and Hannchen, are susceptible. Very few of the varieties found to be resistant have been grown commercially on a large scale. Nearly all of the resistant varieties are of the North African or Coast type of barley or have North African type ancestors. The only barleys of Manchurian origin that appeared resistant are Dorsett and Gatami, which are very similar black-seeded varieties. The variety Feebar appeared to be one of the most highly resistant varieties in the test.

It is evident that many varieties possess resistance to this disease, however, the sources of resistance discovered here may not be very satisfactory to the barley breeder interested in the production of high quality malting varieties of the Manchurian type. It might be a long task to transfer resistance from a low quality variety to a high quality variety. Much time might be saved if a high quality variety resistant to the disease could be found.

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A SIMPLE METHOD USING TRICHLOROACETIC ACID FOR ESTIMATING TOBACCO MOSAIC VIRUS CONCENTRATION[1]

John B. Bancroft and Roy W. Curtis

SUMMARY

A method in which trichloroacetic acid was used for the preparation of virus hydrolysates proved satisfactory for routine assay of tobacco mosaic virus by spectrophotometric methods of measurement at 260 mμ. The method involves precipitation of nucleoprotein from cleared sap at room temperature with 2M trichloroacetic acid and hydrolysis of the precipitate in 0.3M trichloroacetic acid for 15 minutes at 90°C. The technique is relatively rapid and does not require an ultracentrifuge. It is quite selective for virus but not for host products that absorb at 260 mμ and gives results comparable to those obtained by standard methods.

In the development of a screening program for inhibitors of plant virus multiplication, it soon became apparent that if large numbers of samples were to be screened, the routine methods (6, 8, 11) available for the preparation of tobacco mosaic virus (TMV) for spectrophotometric measurement would necessitate the monopolization of an ultracentrifuge. The method of Kirkpatrick and Lindner (2) was found to be unsatisfactory for TMV in tobacco in that the readings obtained from hydrolysates of noninoculated and inoculated leaf discs were nearly equal. Consequently, a new or adapted method that would be suitable for routine use and that would give wide differences between noninoculated and inoculated material had to be developed. The method that was devised is based on the use of trichloroacetic acid (TCA), which is commonly used for protein precipitation and nucleic acid hydrolysis (1, 3, 4, 9). The method does not require an ultracentrifuge but gives results comparable to those obtained from TMV prepared by ultracentrifugation. It is relatively rapid, and it is quite selective for virus but not for host products that absorb at the nucleic acid absorption peak (260 mμ).

MATERIALS AND METHODS.—Tobacco plants (*Nico*

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tiana tabacum L. var. Connecticut Havana 38) were grown under uniform conditions in the greenhouse. In some tests, the leaves of control plants and of plants infected with Johnson's common strain of TMV[2] were harvested, weighed, frozen, and then homogenized in distilled water in a Waring Blendor. In other tests, healthy leaves were removed from the plants and cut along the midribs so that 1 set of half-leaves could be inoculated with virus and the other set rubbed with water. Discs 15 mm in diameter were removed from the leaves with a cork borer, randomized, and cultured on water agar for 6 days under 20-hour photoperiods (1200 foot-candles) at 26.5°C. The discs were then frozen, and samples consisting of 15 discs each were later homogenized in distilled water by means of plastic homogenizers similar to those described by Schlegel and Rawlins (7).

The chloroform-water emulsion technique of Schneider (8) was slightly modified and used for the clearing of sap. A standard 3-minute agitation of the emulsion followed by a 5-minute centrifugation period at 2000 G gave satisfactory results. Four-ml aliquots of the resultant supernatants were removed to acid-washed 10-ml test tubes, to each of which 2 ml of 2M TCA were added at room temperature. A copious white precipitate appeared in the preparations of sap from virus-infected plants at this point, whereas the preparations from healthy plants remained clear or became only slightly cloudy. The tubes were allowed to stand for 5 minutes and then were centrifuged on an Adams safeguard centrifuge at approximately 2000 G for 5 minutes. The supernatants were carefully decanted, and the tubes were drained thoroughly. Six-ml aliquots of 0.3M TCA were added to the tubes, and the samples were hydrolyzed for 15 minutes at 90°C in a thermostatically controlled water bath equipped with a stirrer The hydrolyzed samples were cooled, filtered through Whatman No. 1 filter paper, and measured on a Beckman Model DU spectrophotometer at 260 mμ. It was found to be essential, as discussed by Logan et al (4) that the molarity of acid in the blanks be the same as that used with the plant material and that the blanks be heated the same length of time as the plant samples.

For purposes of comparison, virus nucleoprotein samples were prepared on a Spinco Model L ultracentrifuge. High-speed runs were for 90 minutes at 73,300 G (maximum) and low-speed runs for 15 minutes at 11,730 G (maximum), both in a No. 30 rotor. The optical densities of the preparations were taken at 262 mμ. Local-lesion assays were made by half-leaf inoculations at a dilution of 1:10,000 on leaves of a tobacco variety of Havana type but homozygous for the local-lesion gene from *N. glutinosa* L. The leaves were dusted uniformly with Carborundum and inoculated according to the incomplete block design described by Pound (5).

[2] The authors wish to thank Dr. R. W. Fulton for the TMV strain.

All experiments were run with equal numbers of samples from noninfected and infected plant material. Unless otherwise stated, the results to be presented will be the net difference between readings of preparations from diseased and those of preparations from healthy plants.

RESULTS AND DISCUSSION.—*Extraction of virus from sap.*—To determine if buffer, as compared to distilled water, was necessary for good extraction of virus, aliquots of sap from inoculated and noninoculated plants were divided equally and 1 part of 0.1M phosphate buffer (pH 7.0) was added to 9 parts sap to give a final concentration of 0.01M buffer. Similar dilutions were made with distilled water with the remaining aliquots. Although extraction of buffered sap resulted in a small but significant increase in net absorption readings, it was found that about 10 times as much nonvirus (host) material that absorbed at 260 mμ was extracted from buffered sap as from nonbuffered sap (Table 1). Consequently, all ensuing extractions were made from nonbuffered sap.

An example of the intrinsic standard deviation inherent in the method can be seen in Table 1. In no experiment at any concentration of virus was the standard deviation found to be larger than ± 0.036 optical density units. The results obtained by this method are, therefore, easily reproducible, if the inoculum, tissue, and environmental conditions are standardized.

Conditions for precipitation and hydrolysis.—Experiments undertaken in order to find the most selective TCA molarity for precipitation showed that as molarities were decreased from 2, the readings obtained from noninfected plants increased when expressed as a percentage of readings obtained from TMV-infected plants. A molarity of 4 gave poor results in that the readings were low and the precipitates granular and hard to sediment. In the testing of the effect of the length of time of hydrolysis on yield, maximum readings resulted from hydrolysis with 0.3M TCA for 10 minutes or longer at 90°C. Hydrolysates were scanned at different wave lengths for each time interval of heating. Maximum absorption occurred

TABLE 1.—*Comparison of optical density readings of hydrolysates of precipitates from extracts of nonbuffered and buffered sap from infected and noninfected plants*[a]

Extraction	Optical density[b]		
	TMV sap	Control sap	Net
0.01 M phosphate buffer (pH 7.0)	1.433	0.339	1.094±0.26
Distilled water	1.030	0.035	0.995±.036

[a] Ten samples of each treatment were taken. The net buffer readings were significantly higher than the net water readings at the 1 per cent level of probability.

[b] Optical density (negative $\log_{10}$ of transmission) at 260 mμ.

at 260 mμ for samples heated up to 15 minutes and at 262 mμ for samples heated for 30 minutes. The readings obtained from the virus-infected material hydrolyzed for 15 minutes were indicative of nucleic acid or its products, having an absorption ratio (maximum ultraviolet absorption at 260 mμ/minimum ultraviolet absorption at 230 mμ) of 2.6. Readings of preparations from healthy plants exhibited no peak in the 260-mμ region, and the absorption ratio was less than 1. In addition, the effect of the concentration of TCA used in hydrolysis was tested. Maximum readings were obtained at concentrations of at least 0.2M acid when samples were hydrolyzed at 90°C for 15 minutes. Serial arithmetic dilutions were made of hydrolysates, and the optical densities of the hydrolysates were read. When the data were plotted against dilution, the straight line that resulted indicated the applicability of Beer's law. The results on length of hydrolysis time and on concentration of TCA used in hydrolysis agree with those found in determination of nucleic acid content in animal tissue (3, 9). Experiments with preparations from tomato plants (*Lycopersicon esculentum* Mill. var. Bonny Best), both TMV-infected and healthy, gave results similar to those just described.

Comparison with results of other methods.—In order to compare results from the TCA method with those obtained from ultracentrifuged material and from local-lesion tests, discs were arranged so that harvests of virus and control samples could be made 2, 3, 4, and 6 days after inoculation. The virus concentration in the discs increased in the same fashion whether measured by infectivity, by absorption of virus nucleoprotein prepared by 1½ cycles of ultracentrifugation, or by absorption of TCA-nucleoprotein hydrolysate (Fig. 1).

Calibration of results.—The readings obtained by the TCA method were calibrated in mg virus nucleoprotein/ml in the following manner. The optical density readings of virus and control samples prepared at various dilutions by 1½, and in some cases 2½, cycles of differential ultracentrifugation were taken. From these same preparations, aliquots were removed for determination of virus by the TCA method and for weight determinations. In all cases, the absorbance of purified virus samples/mg virus nucleoprotein was found to be in agreement with the data of Takahashi (10). The optical density readings of the purified virus preparations, however, were twice those obtained with the hydrolysates of the TCA precipitates of the same material (Fig. 2, lines C, A). That is, the TCA hydrolysates, when measured spectrophotometrically,

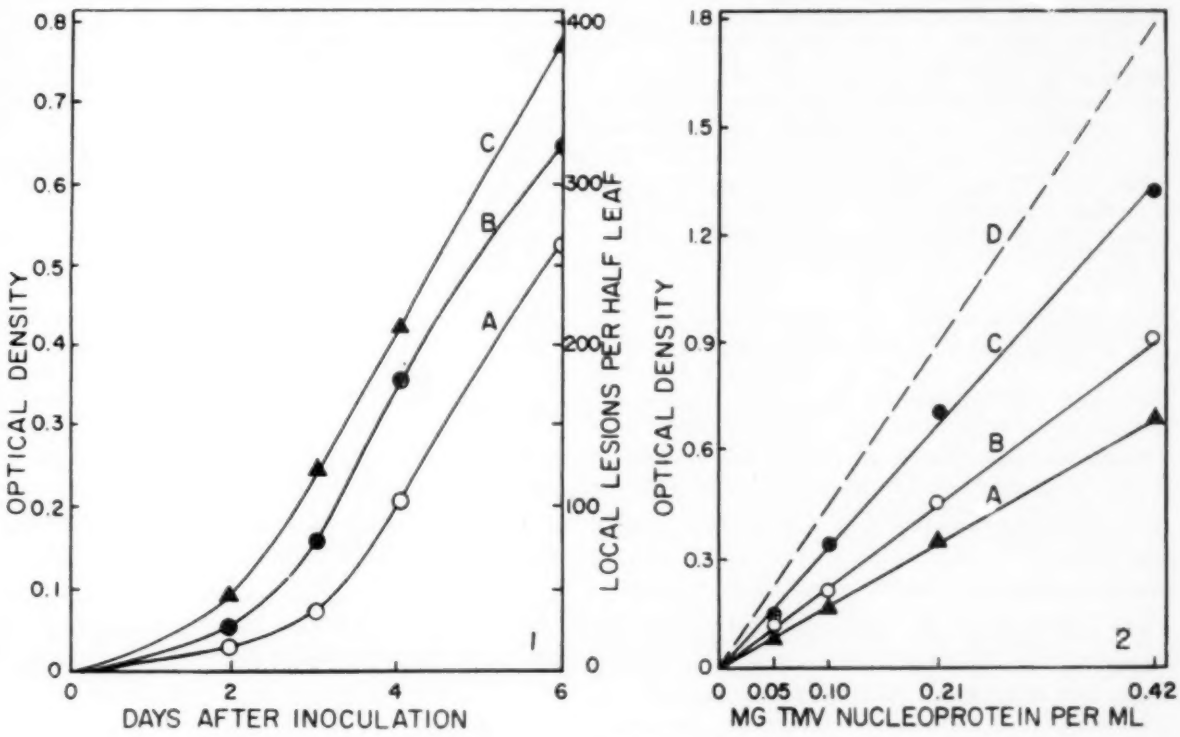

FIG. 1–2. Fig. 1. Comparison of TMV concentrations in tobacco-leaf discs at 2, 3, 4, and 6 days after inoculation. Results were obtained by A) the TCA method, B) ultracentrifugation, and C) local-lesion assay (averages of 9 replicates).—Fig. 2. Ultraviolet absorption of A) the TCA hydrolysates of the TCA precipitates from the ultracentrifuged virus, B) the TCA hydrolysates of the TCA precipitates from cleared sap, C) the virus obtained from the cleared sap by 1½ cycles of ultracentrifugation, and D) the amount of virus calculated as originally present in the cleared sap.

gave only ½ the optical density reading/mg virus nucleoprotein as did the purified virus itself. This ratio was constant when purified samples were used under the conditions previously described.

The next step was to show whether or not both methods, when used with chloroform-cleared sap, gave the same results on a basis of mg virus nucleoprotein/ml. This determination was considered important, for if the optical density readings from the chloroform-cleared sap obtained by the TCA method were more than ½ of those of the virus present in the chloroform-cleared sap, it would mean that something in addition to virus was being assayed. To determine this point, large amounts of chloroform-clarified sap from infected and healthy plants were prepared and diluted so that there would be enough material at each dilution to provide samples for triplicate determinations by TCA hydrolysis and by ultracentrifugation and weighing. It was found that the readings obtained by the TCA method (Fig. 2, line B) were more than ½ of those obtained by ultracentrifugation (Fig. 2, line C). Line C does not represent the actual amount of virus originally present in the chloroform-cleared sap, however, because virus is lost during purification. In order to estimate this loss, various known concentrations of purified virus were again ultracentrifuged and the resulting final concentrations expressed as a percentage of the original concentration. It was found that the loss was 24 per cent; from this value, line D was plotted with line C used as a base line. The final ratios were C:A = 2:1 and D:B = 2:1. These data, in conjunction with those of the previous section, are a strong indication that measurements of the TCA hydrolysates are indeed measurements of material closely associated with, and probably a part of, tobacco mosaic virus.

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NOTICE TO AUTHORS

From this time forward, all manuscripts submitted to PHYTOPATHOLOGY should have journal names abbreviated as listed by CHEMICAL ABSTRACTS. The 1951 Subject Index (Praequine–Z) of CHEMICAL ABSTRACTS (v. 45) contains the most recently published list of such abbreviations. A new listing will appear in the 1956 Subject Index (v. 50), which will be issued within the next 1 or 2 months. Reprints of this list can be purchased from the American Chemical Society.

The *List of Periodicals Abstracted by Chemical Abstracts* is the most widely used and most readily accessible listing available. For these reasons, this change in policy was adopted by the Editorial Board of PHYTOPATHOLOGY at its recent meeting in Cincinnati.

INOCULATION AND RATING METHODS FOR BACTERIAL WILT OF SWEET CORN[1]

John L. Lockwood[2] and Lansing E. Williams

SUMMARY

A reproducible system of rating diseased Golden Bantam corn seedlings was developed. Individual leaves were assigned disease ratings of 0 to 5, based on amount of wilting and on severity and area of lesions. Disease indices were based on pot means derived from leaf ratings. Six-pot replications of 6 plants each generally were sufficient to differentiate between treatments whose disease indices differed by ½ a rating unit or more.

Variation over the range 5×10^5 to 5×10^6 in the numbers of bacterial cells injected into the base of plants did not affect the disease index; coefficients of variation were lowest at the highest inoculum level, however.

The application of a bacterial suspension to the cut ends of clipped seedlings was a highly effective and rapid method of inoculation. Disease severity decreased as the time interval between clipping and inoculation increased. Seedlings in the 2-leaf stage and seedlings clipped below the level of the coleoptile tip were more severely diseased than were younger seedlings or those cut at a higher level. Moisture conditions had little effect on disease severity.

During the early stages of a greenhouse program for the evaluation of chemicals for control of bacterial wilt (Stewart's wilt) of corn (caused by *Bacterium stewartii* E. F. Sm.), the need for methods of attaining a uniform and high degree of disease by rapid inoculation procedures and a reproducible system for rating disease severity became apparent.

The most common method of inoculating corn plants with wilt bacteria has been by introduction of the bacterium into the culm (1, 2, 3) or into the crown and leaf whorl (4, 5, 6) by means of an infested needle or hypodermic syringe. A plant inoculator that permitted the rapid injection of a uniform amount of inoculum into each plant has been described (1). The importance of uniform inoculations in the evaluation of strains of sweet corn for resistance to bacterial wilt has been stressed (1, 2), but no supporting data were presented. Other methods of inoculation that have been used but that have given less satisfactory results than culm or whorl inoculations are 1) atomizing inoculum onto punctured and unpunctured leaves, 2) smearing inoculum on seed, and 3) puncture-inoculating seeds (2). The effect of different levels of inoculum on disease severity and disease variability apparently has not been studied.

Golden Bantam, an open-pollinated variety of sweet corn, has been generally used as a susceptible type in bacterial wilt studies (2, 4, 5, 6).

Indices of disease severity have been derived from ratings of individual leaves (4, 5, 6), and from ratings of individual plants (2). Green weights of plants have also been used as an index of wilt severity (3, 6). In a study of the effect of different corn genotypes on disease reaction, comparisons based on leaf lesion indices were highly correlated with those based on green weights (6). No instance was found in the literature in which the reproducibility of ratings based on an arbitrary scale was tested.

Studies to improve inoculation methods and to test reproducibility of a system for rating disease severity are given in the present report.

MATERIALS AND METHODS.—The culture of *B. stewartii* used throughout these investigations was derived from a single colony obtained by isolation from a seedling Golden Bantam corn plant growing at Wooster, Ohio, in 1954. The culture was maintained on yeast-extract-glucose agar (per liter: yeast extract, 2 g; glucose, 20 g; agar, 20 g) in tubes at 26°C. The isolate was a "virulent" pathogen according to the terminology of McNew (4). The isolate produced severe leaf symptoms on inoculated plants but seldom killed, as was the case with a "highly virulent" isolate.

Sweet corn plants were propagated from seed planted in a steamed mixture of 3 parts Wooster silt loam to 1 part muck.

Inoculum was prepared from 2-day-old slant cultures of *B. stewartii*. The bacteria were suspended in distilled water and the resulting suspension adjusted with an AC model Fisher Electrophotometer (525μ filter) so that light transmission was reduced to 85 per cent. Suspensions so standardized contained approximately 5×10^7 cells per ml.

Plants inoculated by the hypodermic syringe method received about 0.05 ml of suspension (25×10^5 cells) injected into the culm near the coleoptile tip. A tuberculin syringe (B-D Yale No. 1 YT) with a capacity of 1 ml and calibrated in 0.01-ml units was used. Plants were inoculated 10–15 days after planting; at this time they were in the 3- to 4-leaf stage and 4–6 in. high.

A rapid, very effective, and apparently new method was developed for inoculating seedling plants with *B. stewartii*. Plants were clipped with scissors at the

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level of the coleoptile tip and the cut ends swabbed with absorbent cotton that had been dipped in a suspension of the bacterium. Sometimes inoculum was sprayed on the plants. Plants were inoculated 7–9 days after planting, at which time they were in the 1- or 2-leaf stage. After regrowth of the foliage, symptoms in plants inoculated by this method were similar to those in plants inoculated by the hypodermic syringe method.

The method of rating severity of wilt symptoms was similar to those used by McNew (4) and Wellhausen (6) in that individual leaves were rated. At first, attempts were made to rate individual plants after the system of Ivanoff and Riker (2), but lack of uniformity of symptoms among leaves and difficulty in reproducing such ratings necessitated the rating of individual leaves.

The 3 youngest fully expanded leaves on each plant were assigned ratings from 0 to 5. Zero represented a healthly leaf, and 5 a dead leaf. Criteria used in rating disease severity were the amount of wilting and the severity and area of leaf lesions. A leaf with a small chlorotic or necrotic fleck or streak was assigned a rating of ½. Ratings of 1–4 usually were determined by the proportion of the leaf involved by the lesion or group of lesions. In cases of diffuse wilt without discrete lesions, ratings of 2–4 were assigned depending on severity. Ratings of 5 were assigned dead leaves, regardless of whether death was a result of coalescence of several leaf lesions or of systemic wilting.

Mean ratings for plants were used to calculate pot or flat means, the averages of which served as disease indices. Pot or flat means were analyzed by analysis of variance. Plants inoculated by the hypodermic syringe method were rated twice during an experiment, once in the 4- to 5-leaf stage and again in the 5- to 6-leaf stage 5–7 days later. Plants inoculated by the clip method usually were rated once, in the 3- to 4-leaf stage.

The disease indices were sometimes rather low, and disease indices from second or third ratings were often no higher than those from first ratings. The low ratings were due to the facts that the isolate used was not a "highly virulent" one (4) and that the youngest leaf rated was often healthy or only incipiently diseased at the time of evaluation. Because of continued growth of the plant, the 3 youngest leaves at the time of the second or third ratings included 1 or 2 new ones. In some cases these new leaves exhibited only slight symptoms or were disease free. Thus, although the first diseased leaves became more severely diseased with increasing time, this was not reflected by the rating system used in the present study.

RESULTS.—*Reproducibility of disease ratings.*—Because of the great variability among plants in disease symptoms and of the subjectiveness of the rating method, the reproducibility of disease indices was tested by comparing 2 ratings made of the same plants by 1 operator; ratings made by 2 operators on 1 set of plants were also compared. Six-pot replications were used. There were no statistically significant differences between ratings made by the same or different operators (Table 1). Examination of the original data showed that whereas leaf and plant ratings sometimes varied considerably, pot ratings usually did not. It would seem, therefore, that the rating system employed in these studies can be used with confidence.

Replication.—An efficient screening method requires replication sufficient only to discover the desired magnitude of difference. A pot mean of disease severity was determined by rating 3 leaves from each of 6 plants in a pot. The total of 18 ratings was divided by 18 and expressed as a mean. Estimates of variance of this mean were calculated with data from 36 separate 6-pot experiments. The mean estimate of variance, 0.189, was used as an estimate of the true variance. Alpha (risk of rejecting a true hypothesis) was set at 0.05, and beta (risk of accepting a false hypothesis) at 0.10. When these values were used, the difference from a true mean that could be detected was calculated to be 0.565. Generally then, 6-pot replications would differentiate between treatments whose disease indices differed by ½ a rating unit or more.

Since a 50 per cent increase in replication (9 pots) would increase the precision by only 18 per cent (a difference of 0.461 unit), and one of 100 per cent (12 pots) would increase it only 29 per cent (0.399 unit), 6-pot replications were considered most efficient and were used routinely in later chemical-control studies.

Variety.—Although Golden Bantam has been commonly used in bacterial wilt studies (2, 4, 5, 6), it was thought that a hybrid might be more uniform with respect to disease susceptibility. Therefore, North Star, a hybrid, was compared with Golden Bantam in an inoculation experiment. Plants were inoculated by the hypodermic syringe method and rated for disease at 3 dates. Twenty pots were used for each type. The 2 types did not differ significantly in

TABLE 1.—*Disease indices of plants inoculated with Bacterium stewartii and rated by 2 operators*

Test	Days after inoculation	Operator	Indices[a] of plants given treatments indicated			
			1	2	3	4
1	12	1	1.4	1.3	2.5	2.1
1	12	2	1.3	1.3	2.3	2.1
2	6	1	1.4	1.2	1.4	1.1
2	6	2	1.3	1.2	1.3	1.1
2	11	1	1.4	1.4	1.3	1.1
2	11	2	1.3	1.4	1.3	1.1

[a] Each index is the mean of ratings of 6 plants in each of 6 pots; 3 leaves of each plant were rated on a scale of 0 to 5, with 0 indicating no disease and 5 indicating a dead leaf. Differences between operators were not statistically significant; there were no statistically significant interactions. Each test was analyzed separately.

TABLE 2.—*Disease indices and coefficients of variation for 2 sweet corn varieties inoculated by hypodermic syringe method with Bacterium stewartii*

Days after inoculation	Golden Bantam		North Star	
	Disease index[a]	Coefficient of variation	Disease index[a]	Coefficient of variation
6[b]	2.0	18.9	2.1	20.2
12[c]	1.7	28.6	2.5	34.1
18[c]	3.3	19.1	4.0	19.6

[a] Each index is the mean of ratings of 6 plants in each of 20 pots; 3 leaves of each plant were rated on a scale of 0 to 5, with 0 indicating no disease and 5 indicating a dead leaf.
[b] Differences between disease indices were not statistically significant.
[c] Differences between disease indices for varieties were statistically significant at the 1 per cent level.

disease index 6 days after inoculation (Table 2); North Star was the more severely diseased after 12 and 18 days. The coefficient of variation was slightly higher for North Star at all 3 rating dates, with the highest coefficients of variation for both types occurring after 12 days. Thus, there appeared to be no advantage in using the hybrid.

Inoculation by hypodermic syringe method.—Tests were made to determine if varying amounts of inoculum might contribute to lack of uniformity of disease symptoms among plants inoculated by hypodermic injection, as suggested by previous workers (1, 2). Suspensions containing 1×10^7, 3×10^7, and 1×10^8 bacterial cells per ml were injected into plants, the volume of suspension being kept constant at 0.05 ml. As a comparison, plants injected with nonuniform volumes of inoculum were also included. Ten-pot replications were used.

There were no significant differences among disease indices of plants given the different levels of inoculum nor between indices for plants inoculated with uniform and nonuniform amounts of inoculum (Table 3). The lowest coefficients of variation, however, were obtained at the highest inoculum level used.

A comparison also was made between the severity of disease in plants inoculated by 2 different operators. In these experiments, care was not taken to inject constant volumes of inoculum. The inoculum suspension contained approximately 5×10^7 cells per ml. Twenty-pot replications were used. There were no statistically significant differences between disease indices of plants inoculated by different operators.

TABLE 3.—*Disease indices and coefficients of variation of plants injected with different concentrations of inoculum of Bacterium stewartii*

Volumes of suspension (ml)	Number of cells per ml	Disease index[a]		Coefficient of variation	
		6 Days	11 Days	6 Days	11 Days
0.05	1×10^7	1.3	1.4	16.0	26.3
0.05	3×10^7	1.3	1.3	16.2	27.0
0.05	1×10^8	1.3	1.3	14.3	15.8
0.01–0.10[b]	5×10^7	1.1	1.2	19.0	25.0

[a] Each index is the mean of ratings of 6 plants in each of 6 pots; 3 leaves of each plant were rated on a scale of 0 to 5, with 0 indicating no disease and 5 indicating a dead leaf. Differences between disease indices were not statistically significant.
[b] Plants inoculated without regard to exact volume.

The volume of inoculum was controlled in further inoculations; 0.05 ± 0.01 ml of a suspension standardized to contain 1×10^8 cells per ml was used per plant.

Clip method of inoculation.—In order to determine optimum conditions for disease development in plants inoculated by the clip method, tests were made of 4 variables: 1) stage of development of plant at inoculation, 2) level of clipping, 3) moisture conditions, and 4) time interval between clipping and inoculation. Stage of development and clipping level were tested together as were moisture and time interval. Plants were grown in flats. Three replications of 25–30 plants each were used.

Variability in rate of germination made it possible to classify plants into 3 stages of development: 1) coleoptile stage, with coleoptile unruptured or first leaf emerging slightly; 2) first-leaf stage, with first leaf partly unrolled; and 3) second-leaf stage, with second leaf partly unrolled.

Plants of all 3 stages were clipped at 3 levels: 1) ½ in. below, 2) at the level of, and 3) ½ in. above the coleoptile tip.

Tests were made of 3 moisture conditions: 1) flats with soil moist throughout the experiment, 2) flats with soil not watered from 24 hours before inoculation until 24 hours after inoculation, and 3) flats placed in moist chamber for 24 hours after inoculation.

Plants were inoculated after the following time intervals following clipping: 1) immediately, 2) 4 minutes, 3) 16 minutes, 4) 1 hour, and 5) 4 hours. Clipping times were so adjusted that all plants were inoculated at approximately the same time.

There was a marked decrease in disease index with lengthening interval between clipping and inoculation (Table 4). A delay of only 4 minutes between clipping and inoculation resulted in a decrease in disease. There was only a trace of disease when the interval was 4 hours. The decrease in disease index with increasing time interval was due primarily to a decrease in severity of symptoms, but there were slight decreases in disease incidence.

Moisture conditions influenced the amount of disease to a lesser extent than did interval between clipping and inoculation (Table 4). Disease index in dry soil was higher (5 per cent level of significance) than in wet soil in 1 experiment. In another experiment, disease index of plants placed in the moist chamber was higher (5 per cent level of significance) than that of plants in dry soil. Incidence of disease was almost the same for all moisture conditions in both experiments. There was no interaction between time interval and amount of moisture.

Stage of development of plants at the time of inoculation influenced amount of disease (Table 5). Dis-

TABLE 4.—*Disease indices of plants inoculated with Bacterium stewartii by clipping method at different time intervals and under different moisture conditions*

Moisture conditions	Disease index[a] of plants with interval between clipping and inoculation as indicated					
	0 Minutes	4 Minutes	16 Minutes	60 Minutes	240 Minutes	Mean
Dry soil	1.40	0.97	0.91	0.52	0.26	0.81
Wet soil	1.21	0.84	0.57	0.33	0.14	0.62
Moisture chamber[b]	1.56	1.01	0.64	0.41	0.19	0.76
Mean	1.39	0.94	0.71	0.42	0.20	

[a] Each index is the mean of ratings of 25–30 plants in each of 3 replications; 3 leaves of each plant were rated on a scale of 0 to 5, with 0 indicating no disease and 5 indicating a dead leaf. LSD for moisture condition means at the 5 per cent level was 0.15; at the 1 per cent level, 0.20. LSD for time interval means at the 5 per cent level was 0.19; at the 1 per cent level, 0.26. There were no significant interactions.
[b] Wet soil; flats were placed in moist chamber for 24 hours.

ease index was highest in plants in the first- and second- leaf stages. Plants clipped at the lowest level had a higher disease index than did plants clipped at the 2 higher levels. Maximum disease was present, therefore, in plants of intermediate and older stages clipped at the lowest level. Incidence of disease, which was about 90 per cent, was not affected by stage of development or clipping level. Differences in disease indices, then, were due to differences in severity of symptoms. There was no interaction between stage of development and clipping level.

Inoculation could be done as effectively by spraying with bacterial suspensions as by dabbing each plant with infested cotton. Disease incidence and severity of symptoms did not differ for the 2 methods.

DISCUSSION.—In any general screening program, efficiency of operation as well as uniformity and reproducibility of results are of concern. The experiments described in this report were designed to improve and standardize inoculation and disease rating methods for bacterial wilt of sweet corn for use in subsequent greenhouse testing of chemicals for control of this disease. The methods described might also be used in other research with this disease, e. g. studies on wilt resistance.

The disease rating system used was sufficiently precise to detect differences of ½ a rating unit between treatments. This degree of precision was considered adequate and most efficient for purposes of the testing program. The relatively large increase in replication necessary to increase substantially the sensitivity of the tests did not appear warranted.

TABLE 5.—*Disease indices of corn plants clipped at different levels and stages of development, then inoculated with Bacterium stewartii*

Stage	Index[a] of plants clipped at indicated level			Mean
	Below coleoptile tip	Coleoptile tip	Above coleoptile tip	
Coleoptile	1.45	0.60	0.51	0.85
First leaf	2.36	1.39	0.90	1.55
Second leaf	2.82	1.57	1.83	2.07
Mean	2.21	1.19	1.08	

[a] Each index is the mean of ratings of 25–30 plants in each of 3 replications; 3 leaves of each plant were rated on a scale of 0 to 5, with 0 indicating no disease and 5 indicating a dead leaf. LSD for stage or clipping level means at the 5 per cent level was 0.38; at the 1 per cent level, 0.52. There was no significant interaction.

Over the concentration range tested, the uniformity of disease symptoms in plants inoculated by hypodermic injections was found to be influenced to a greater extent by the concentration of the bacterial cells in the inoculum suspension than by the uniformity of the volume injected. These results are somewhat at variance with the opinions of other workers (1, 2), who emphasized the importance of uniform inoculations. Under conditions different from those used in the present study, uniformity of inoculation might be of greater importance in obtaining uniform disease. The disease index was not affected either by the level of inoculum or the uniformity of inoculation. It is possible that if low concentrations of bacterial cells had been used, differences in disease index would have occurred. High levels of inoculum (5×10^6 cells per plant) would be desirable, due to the reduction in variability of disease with such suspensions.

The clip method of inoculation is apparently an innovation for bacterial wilt of corn and for bacterial diseases of other plants as well. This method is highly effective and can be used with great speed. One operator can easily clip and swab 100 plants per minute. The operation can be accelerated by use of 2 operators, 1 to clip and 1 to inoculate, or by spray-inoculating. It is not inconceivable that large-scale inoculations could be made in the field by combined use of a mower and sprayer. The clip method might be employed effectively in the evaluation of sweet corn strains for resistance to bacterial wilt. As disease severity was greatly affected by the time interval between clipping and inoculation, and by the age of seedling and level of clipping, standardization of these factors would be important for critical work.

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STUDIES ON THE ETIOLOGY AND EPIDEMIOLOGY OF DIAPORTHE PHASEOLORUM VAR. CAULIVORA, THE CAUSE OF STEM CANKER OF SOYBEANS[1]

F. I. Frosheiser[2]

SUMMARY

Plants of susceptible soybean varieties were inoculated with ascospores and conidia of *Diaporthe phaseolorum* (Cke. & Ell.) Sacc. var. *caulivora* Athow & Caldwell. No stem canker symptoms developed although the pathogen was reisolated from numerous stems of inoculated plants.

Inoculations made by inserting toothpicks infested with mycelium of the fungus into the stem generally caused typical symptoms of the disease. Varieties appearing resistant to natural infection were readily infected by this method of inoculation.

Pycnidia with viable conidia were produced by the fungus on autoclaved stems of sweetclover, red clover, alfalfa, wheat, rye, oats, barley, lambsquarters, and rough pigweed; and also on agar containing finely ground sweetclover leaves and stems or wheat straw. Ground sweetclover leaves in 1.5 per cent agar was the best substrate for producing pycnidia and conidia.

Stem canker caused a loss in yield of 10.1 bushels per acre when Blackhawk soybean plants 70 days old were inoculated with mycelium; 67 per cent of the plants were infected. Inoculation of 84-day-old plants caused no significant reduction in yield.

Sixteen varieties of soybeans were compared for susceptibility to natural stem canker infection. Renville, Blackhawk, and Capital contained 7.7, 5.0, and 2.9 per cent infected plants, respectively, whereas Pridesoy 11, Ottawa Mandarin, and Flambeau showed no symptoms.

No symptoms of stem canker developed on soybeans planted in artificially infested soil in the greenhouse or field. Seedlings were infected in test tubes, and some were killed. Some infected seedlings recovered and grew to maturity without symptoms after being transplanted into autoclaved soil in the greenhouse. There was no evidence that infection of older plants was due to seedling infection or to the planting of infected seed.

Crimson clover, Ladino clover, red clover, sweetclover, alfalfa, snap beans, and garden peas were infected in the greenhouse following inoculation with mycelium of *D. phaseolorum* var. *caulivora*.

The only known method of survival of the pathogen is in overwintered plant parts and seed. It is possible that the pathogen lives saprophytically in the soil or survives in plant material other than soybean.

Stem canker, caused by *Diaporthe phaseolorum* (Cke. & Ell.) Sacc. var. *caulivora* Athow & Caldwell is one of the most destructive diseases on soybean (*Glycine max* (L.) Merr.) in the north central states. The term "stem canker" was first suggested for this disease by Crall (5) in 1950. The history of stem canker is difficult to trace, because for a long time its symptoms were included in the disease complex known as pod and stem blight.

Welch and Gilman (18) reported that the fungus causing stem canker differed in morphology and pathogenicity from the pod and stem blight fungus, *D. phaseolorum* (Cke. & Ell.) var. *sojae* (Lehman) Wehm. Athow and Caldwell (3) confirmed Welch and Gilman's statement and named the organism *D. phaseolorum* var. *caulivora*.

Stem canker is distributed throughout the corn belt

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Cooperative investigations of the Field Crops Research Branch, ARS, USDA, and the Minnesota Agricultural Experiment Station.

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of the United States and has been reported from Ontario, Canada (1, 3, 4, 5, 11, 15). There is no evidence in the literature that this disease has been found outside the United States and Canada. Estimates up to 80 per cent infected plants in some fields have been reported (6, 9, 12). Yield losses due to stem canker have been estimated as high as 50 per cent (1).

Stem canker was first reported from Minnesota by Kernkamp and Gibler (15) in 1951, at which time they observed it in 4 counties. In 1952 it was generally prevalent in the soybean-growing area of the state, with infections as high as 7 per cent in some fields. In 1953 approximately the same amount of infection was observed, and in 1954 less than 1 per cent of the plants were infected in all fields in which stem canker was found.

The disease usually appears in August, although it has been reported to occur as early as the middle of July (14). Prior to 1954, the disease had not been observed in Minnesota earlier than the last week of August, but in 1954 the first naturally occurring symptoms were seen in a field near Rosemount, Minnesota, on August 6, at which time some infected plants were already dead.

Because the disease appeared potentially destructive in Minnesota, a study of the etiology and epidemiology of the stem canker fungus was begun in 1952. The results of these investigations are presented here.

MATERIALS AND METHODS.—All isolates of *D. phaseolorum* var. *caulivora* used were originally isolated from cankers on diseased soybean plants collected from commercial fields in Minnesota. Mass isolates were used during the first year, whereas monoascospore or monoconidial isolates were used either individually or in mixtures during the second year.

Inoculations were made by the toothpick method (7) by inserting the infested toothpick point into the stem through the leaf scar of a lower node.

Ascospore inoculations were made with ascospore suspensions prepared by growing the fungus on autoclaved soybean stems until abundant perithecia developed. The perithecia were scraped off the stems and crushed in sterile distilled water. The resulting material was strained through a sintered glass filter to remove plant and perithecial material as well as most of the mycelium. The spore suspension contained at least 30,000 spores and asci per ml. The ascospore suspension was either sprayed on the plants, applied to the injured or uninjured leaf scars, or injected into the stems with a hypodermic syringe.

Conidial suspensions were made by growing the organism on 1.5 per cent agar containing 20 g of finely ground sweetclover leaves and stems per liter. After abundant conidial ooze appeared on the pycnidia, enough sterile distilled water was added to cover the surface of the culture. The petri plates were agitated to dislodge and suspend the conidia. The suspension was decanted and diluted as with the ascospore suspension. Conidial suspensions were applied to the plants in the same manner as were the ascospore suspensions.

Greenhouse tests were made in 2 greenhouses, one operating at 23°±1 and the other at 18°C±1. In either greenhouse the temperature sometimes rose 6° higher than the regulated temperature for brief periods on sunny days.

Moist chambers used for infection in the greenhouse consisted of a wooden frame covered with sheet plastic. Moisture was introduced constantly into the chamber in the form of a fine mist produced by a mechanical humidifier.

Blackhawk soybeans, known to be susceptible to stem canker, were used in all tests of methods of inoculation and yield reduction, except where otherwise stated. Varietal-susceptibility studies included all varieties commonly grown commercially in Minnesota, varieties recently introduced or developed by seed companies, and new varieties or promising selections developed in the cooperative program of the U. S. Regional Soybean Laboratory and cooperating Agricultural Experiment Stations.

Isolations made from inoculated plants to determine if infection had taken place were made by surface disinfesting stem tissue in a 1 per cent sodium hypochlorite solution for at least 1 minute, then plating the material on acidified potato-dextrose agar.

When age of plants is mentioned it refers to the time elapsed since the seed was planted.

RESULTS.—*Perithecial production.*—The symptoms of stem canker of soybean and the morphology of the sexual stage of *D. phaseolorum* var. *caulivora* have been described adequately by Athow and Caldwell (3). Very little is known about the survival of the organism or its occurrence on material other than soybean. To obtain more information on the survival, extent of saprophytic growth, and sporulation of the organism, a study was made to determine if its growth and sporulation were limited to soybean plant residue.

Autoclaved stems of 10 crop plants and 5 weed species were infested with a mixed ascospore suspension of 4 isolates of *D. phaseolorum* var. *caulivora* and incubated in 125-ml Erlenmeyer flasks in the laboratory. The fungus made a vigorous mycelial growth on all the stems. Four weeks after inoculation, some perithecia were formed on all the material tested, but they were most numerous and nearly equal in number on soybean (*Glycine max* L.), alfalfa (*Medicago sativa* L.), barley (*Hordeum vulgare* L.), flax (*Linum usitatissimum* L.), and lambsquarters (*Chenopodium album* L.); intermediate in numbers on red clover (*Trifolium pratense* L.), white sweetclover (*Melilotus alba* Desr.), corn (*Zea mays* L.), rye (*Secale cereale* L.), oats (*Avena sativa* L.), barnyard grass (*Echinochloa crus-galli* (L.) Beauv.), and rough pigweed (*Amaranthus retroflexus* L.); and few on witch grass (*Panicum capillare* L.), wheat (*Triticum aestivum* L.), and green foxtail (*Setaria viridis* (L.) Beauv.).

The asexual stage.—The writer has observed no pycnidia of *D. phaseolorum* var. *caulivora* on soybean stems or on potato-dextrose agar. In a few instances,

true conidia have been observed oozing from ruptured stromata on potato-dextrose agar. Timnick et al (17) reported alpha conidia oozing from stromatal bodies on autoclaved soybean stems infested with this fungus. They also observed small pycnidia, containing numerous beta spores and an occasional alpha spore, being produced on a number of media. Evidently they did not find pycnidia producing a considerable number of alpha spores. Athow and Caldwell (3) concluded that the conidial or imperfect stage of this fungus is lacking. Welch and Gilman (18) were unable to find the asexual stage of this fungus. Frosheiser and Kernkamp (10) reported the occurrence of pycnidia with conidia on a number of autoclaved plant materials. Hildebrand (13) has since reported the occurrence of conidia born in ectostromatic bodies and on mycelium.

Pycnidia containing alpha conidia were observed on 9 of the 15 species of plants in the experiment mentioned above, in which various autoclaved plant stems were infested with ascospores of *D. phaseolorum* var. *caulivora*. Pycnidia were most numerous on stems of sweetclover and relatively abundant on wheat, rye, oats, barley, alfalfa, red clover, and lambsquarters; they were very scarce on rough pigweed, and were not found on soybean, flax, barnyard grass, witch grass, or green foxtail. The pycnidia and conidia were of the Phomopsis type.

Monoconidial isolates from sweetclover stems produced typical mycelium and perithecia of *D. phaseolorum* var. *caulivora* on potato-dextrose agar. Mycelium from 1 monoconidial isolate was used to inoculate Blackhawk soybean plants in the field, and typical stem canker symptoms developed on all of the 19 plants inoculated.

Three monoconidial and 2 monoascospore isolates were compared on potato-dextrose agar and on finely ground wheat straw, sweetclover leaves, and sweetclover stems, each in 1.5 per cent agar. Perithecia were formed abundantly by all isolates on potato-dextrose agar. No pycnidia were observed in any of the isolates on this medium; however 1 isolate produced conidia that oozed from a ruptured stroma. On wheat- and sweetclover-stem agar, pycnidia and perithecia were produced in approximately a 1:1 ratio. On sweetclover-leaf agar, no perithecial beaks were observed, but pycnidia were relatively abundant and a few stromatal bodies in 1 isolate also contained conidia.

Sparse mycelial growth was observed on sweetclover-leaf agar. The pycnidia were of the Phomopsis type and were single, black, round, and slightly flattened above. The diameter of the pycnidia ranged from 198 to 528μ. Conidia were liberated from the pycnidia in a slimy, cream-colored exudate that formed globules on the pycnidia (Fig. 1).

The conidia were hyaline and nonseptate and contained 2 guttulae per spore (Fig. 2). They measured 2.4–8.3 $\times$ 1.5–3.9μ. The writer has not observed the production of stylospores, also known as beta conidia. It is possible that they were overlooked or that the proper conditions for their formation were lacking.

Infection tests.—Soybean breeders are interested in developing varieties that are resistant to stem canker as well as having other desirable characters. At present there is no reliable method for inducing stem canker in a large number of plants in a short time,

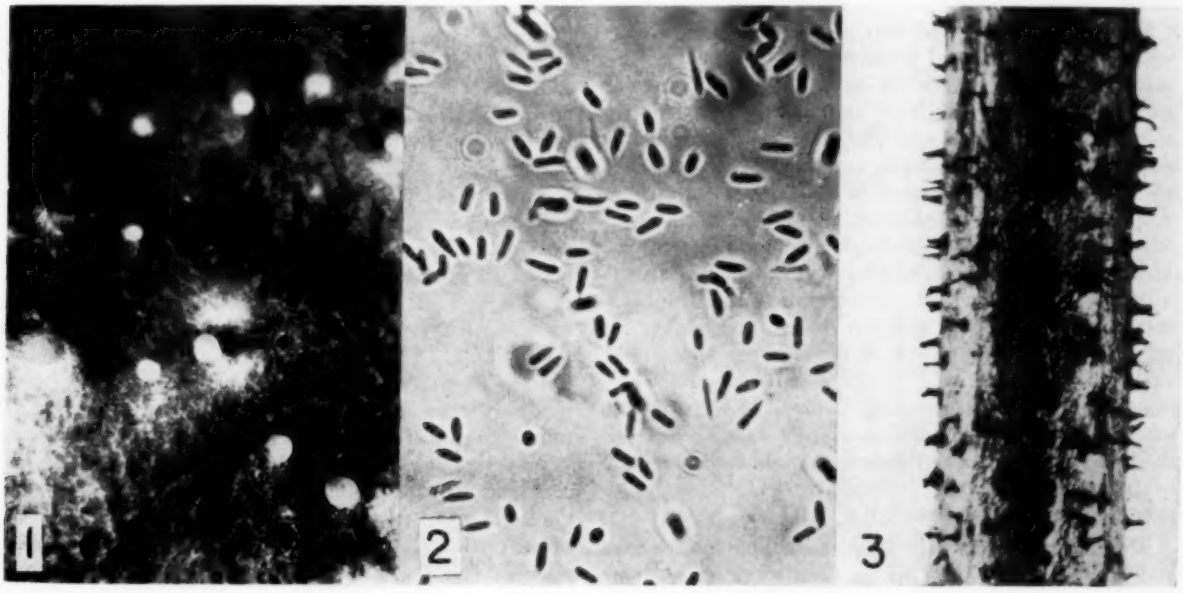

FIG. 1-3. Fig. 1. Spore masses extruded from pycnidia of *Diaporthe phaseolorum* var. *caulivora* on sweetclover-leaf agar.—Fig. 2. Conidia of *D. phaseolorum* var. *caulivora*.—Fig. 3. Perithecia formed on alfalfa stem artificially infected with *D. phaseolorum* var. *caulivora*.

so that resistant material may be screened. The toothpick method of inoculation is a positive method of inducing stem canker in soybean plants, but breeders generally consider it too severe and too time consuming for use in large-scale breeding programs.

The manner by which natural infection occurs is not known. It has been suggested that the fungus enters the leaf or petiole and grows to the main stem or branch, where cankers form at the juncture of the petiole and branch or stem. The idea presumably is based on the fact that cankers begin at the leaf scar on naturally infected plants. It also has been suggested that infections occur through the leaf scar.

Methods of inoculation that would more nearly duplicate the manner in which natural infections probably occur therefore were tried. Ascospores and conidia of *D. phaseolorum* var. *caulivora* were used for the inoculation of plants both in the greenhouse and field. In the greenhouse, ascospore and conidial suspensions were applied to injured and uninjured leaf scars near the base of Blackhawk soybean plants, and the plants were incubated 48 hours in a moist chamber at 18° and 23°C, after which they were transferred to greenhouse benches at the same temperature. Cotton gauze was soaked in a conidial or an ascospore suspension and wrapped around a lower node of the plants. This was covered with grafting tape to keep the spores moist. Other plants were inoculated by injecting the spores directly into the stem by means of a hypodermic syringe. Ascospore suspensions were sprayed on the plants, which were afterward incubated in moist chambers. In several of the tests, all conditions were identical except that ascospores were used in one case and conidia in another. Ages of plants at the time of inoculation ranged from 42 to 92 days. The inoculated and control plants were allowed to grow to maturity.

In no case did typical stem canker symptoms occur when spores were used as inoculum. Portions of the mature plant stems near the point of inoculation were surface disinfested in a 1 per cent sodium hypochlorite solution for 1 minute and plated on acidified potato-dextrose agar. The pathogen was recovered from many of the stems, showing that it had entered the host tissue. Evidence of infection, therefore, was based on recovery of the fungus from the inoculated stems. There was little difference in infection rates obtained with the different methods of inoculation or with different temperatures. The greatest difference in infection rate occurred among plants of different ages, where it varied from 16 per cent of the plants infected when inoculated with ascospores at 42 days to 61 per cent when the plants were inoculated at 92 days. Wherever the infection produced by ascospores and conidia could be compared directly, the conidial inoculations always produced the greater amount of infection.

Field inoculations were made with both ascospores and conidia. Ascospore suspensions were sprayed on injured and uninjured Blackhawk soybean plants 49, 64, 79, and 91 days old, and the plants kept moist by covering with plastic tents for 36 hours. No stem canker symptoms appeared on these plants. Scattering perithecial material between the soybean rows at 2-week intervals during the growing season or injecting ascospores into the stems produced no symptoms. Both ascospores and conidia were applied to injured and uninjured leaves and petioles of 10-week-old Blackhawk soybean plants. The leaves and petioles were covered with cellophane bags for at least 72 hours following inoculation. Both types of spores were applied at the juncture of a lower petiole and stem and covered with sheet plastic for 3 days. One plant developed a small canker at the node where it was inoculated. No other symptoms developed from these inoculations. At maturity, the portion of the stem where the petiole had been attached was surface disinfested and plated on acidified agar. One of 38 plants inoculated with ascospores (spores applied to an injured petiole) yielded the pathogen on reisolation. The pathogen was recovered from 5 of 29 plants inoculated with conidia; 3 of these were from node inoculations, and 2 were from injured petiole inoculations. The pathogen was not recovered from the controls, where distilled water was used instead of the spore suspensions.

Seedling infection.—In a disease of this nature, the possibility of systemic invasion arising from seedling infection cannot be overlooked.

In 1953 a replicated field plot was planted with 6 varieties of soybeans, including Blackhawk. Inoculum, consisting of mycelium of *D. phaseolorum* var. *caulivora* grown in a rice-vermiculite mixture, was spread over the seed before it was covered with soil. No infection and no reduction of stand were observed. In a similar test, Blackhawk soybeans were planted in the greenhouse in autoclaved soil that had been infested with the organism. No stem canker symptoms developed.

Following these results, tests were made to determine if the organism could attack very young seedlings under the most severe conditions. Seeds of the Blackhawk variety were surface disinfested in a 1:1000 mercuric chloride solution for 1 minute, rinsed in a 1 per cent sodium hypochlorite solution for 1 minute, and then placed on potato-dextrose agar slants, 1 seed per test tube. Seven of these were inoculated with mycelium of each of 2 isolates of *D. phaseolorum* var. *caulivora*. Four were not inoculated and were used as controls. They were held in the laboratory at approximately 24°C. The seeds began to germinate normally, but the portion of the radicle below the mycelial mat gradually turned brown and rotted. A normal primary root system was not formed, and the inoculated seedlings grew slowly compared to the controls, in which a normal root system developed. At the end of 7 days, the roots of the inoculated seedlings were completely brown below the mycelial mat. Twelve days after planting, all the seedlings except 2 were recovering, having produced adventitious roots. Mycelium had completely overgrown the 2 dead seedlings. Five of the recovering seedlings plus 2 controls were trans-

planted into autoclaved soil in the greenhouse at 23°C. All the transplanted seedlings continued to develop normally to maturity. The inoculated seedlings, smaller from the beginning, remained smaller than the controls, undoubtedly because of the injured primary root system. Roots and stems of the mature plants were plated on acidified potato-dextrose agar, and the organism was not recovered from the inoculated plants or from control plants.

It was evident that *D. phaseolorum* var. *caulivora* could infect and even kill very young seedlings, but its progress was arrested in surviving seedlings and did not produce symptoms in the developing plants.

Natural seed infection.—Surface-disinfested seeds from infected soybean plants collected in 1953 were plated on acidified potato-dextrose agar to determine the extent of infection in seed from infected plants. The seeds were placed in the following categories: 1) seeds from pods formed on or within 2 in. of the canker; 2) seeds from the remaining portions of the plants in group 1; 3) bulk seed from infected plants in a plot at the University farm, containing 5 per cent infected plants; 4) bulk threshed seed from the plot in 3.

In group 1, 5.1 per cent of 331 seeds yielded the fungus. The fungus was not recovered from 557 seeds of group 2, nor from 600 seeds of group 4. One colony of the fungus was obtained from 636 seeds in group 3.

Infected seed may be a source of inoculum for the spread of this disease, but systemic infection of the plants, due to planting infected seed, does not appear to be an important factor. Hildebrand (12) planted seed from infected and noninfected plants. Seed from infected plants produced plants of which 6.8 per cent had stem canker, whereas 6.1 per cent of the plants from seed from healthy plants had stem canker. If the seedling is killed early or the spread of the infection is arrested in the seedling stage, adult plant infection from infected seed is unlikely.

When seed was plated on acidified agar, most infected seed did not germinate, whereas noninfected seed germinated very readily. A few infected seed began to germinate but were killed before the radicle attained a length of 1.5 cm.

Yield reduction studies.—Estimates of loss due to stem canker in commercial fields have been discussed. Athow and Caldwell (3) reported 60 per cent loss in seed weight when comparing seed from 100 healthy plants with seed from 100 plants killed by stem canker. The writer undertook a similar study in 1953. Thirty plants killed by stem canker and 30 apparently healthy plants were selected from a field of soybeans. The pods were harvested separately from each plant and counted. The seed from each plant was carefully threshed, counted, and weighed. The total weight of seed from healthy plants was 13 per cent greater than that from plants killed by stem canker. The average weight per seed was 22 per cent greater from the healthy plants.

A yield-reduction trial was made to determine the effect of different rates and dates of inoculation on the yield of seed. A split-plot design was used in which the rates of inoculation were the main plots and the planting dates the subplots within the main plots. Each plot consisted of 2 rows, 10 ft. long and spaced 40 in. apart, which were reduced to 8 ft. in length prior to harvest. The plants were thinned when approximately 6 in. tall to a uniform spacing of 50 plants per 8 ft. of row. The plants in 1 row of each plot were inoculated with the toothpick method, and sterile toothpicks were inserted into the stems of a corresponding number of plants in the other row. Two rates of inoculations were used: all the plants in a row and 30 per cent of the plants in a row. Inoculations were made 70 and 80 days after planting. The entire experiment was randomized and replicated 4 times.

The results of the trial are summarized in Table 1. A statistically significant reduction in yield of 10.1 bushels per acre occurred where all of the plants were inoculated 70 days after planting; 67 per cent of such plants became infected. The reduction in yield in any of the other treatments was not statistically significant. The results indicate that early infections are much more destructive than later infections.

Survival of the organism.—The stem-canker fungus is known to survive in overwintered soybean stems and in infected seed (18). Soybean stems, affected by stem canker, were collected in the fall of 1953. A portion of these was covered with 1 in. of soil, and the re-

TABLE 1.—*Yield of Blackhawk soybeans and percentage of infected plants in field plots in which different percentages of the plants were inoculated, when 70 or 80 days old, with Diaporthe phaseolorum var. caulivora by the toothpick method*

Percentage of plants inoculated	Age at inoculation (days)	Percentage of plants infected	Percentage of plants killed	Yield,[a] bushels/acre	
				Inoculated rows	Control rows
100	70	67.0	41.0	25.8**[b]	35.9
100	84	80.0	5.5	35.6	36.3
30	70	13.0	4.5	34.6	34.9
30	84	27.5	5.5	34.9	37.0

[a] Based on yield of 4 rows, each 8 ft. long.

[b] Analysis of variance of the difference in yield between inoculated and noninoculated rows, by the split-plot method, indicated that this yield was significantly lower at the 1 per cent level, than that for any other treatment.

mainder was lightly covered with trash, consisting of dry grass and tree leaves, outdoors in St. Paul. The following spring and summer they were examined periodically.

A few fully developed perithecia with ascospores were first observed on the stems covered with soil, on May 24. On May 29, the perithecia were quite numerous on the soil-covered stems, and on June 2 the first perithecia with ascospores were observed on stems covered with trash. Perithecial development reached its peak on both the soil- and trash-covered stems by July 5. The dry weather conditions early in the spring undoubtedly delayed perithecial development on the trash-covered stems. The stems under the soil were continually moist whereas those under trash usually were dry when examined prior to the latter part of May.

Some investigators are of the opinion that the stem-canker fungus is endemic in some soils (2, 12). The writer has attempted, without success, to isolate the fungus from soil taken from a field in which stem canker had appeared the previous season. In view of the fact that stem canker appears in some fields that have never been planted to soybeans, as has been observed here and by others (2, 13), it appears that the fungus is either soil-borne or has hosts other than soybeans.

Host range studies.—A limited study was undertaken to determine if *D. phaseolorum* var. *caulivora* could infect other leguminous plants. Plants of garden pea (*Pisum sativum* L.), crimson clover (*Trifolium incarnatum* L.), Ladino clover (*T. repens* L.), red clover (*T. pratense* L.), alfalfa (*Medicago sativa* L.), white sweetclover (*Melilotus alba* Desr.), snapbean (*Phaseolous vulgaris* L.), and soybean were inoculated in the greenhouse. Mycelium-infested toothpicks were inserted into the base of the stem. Peas, beans, alfalfa, sweetclover, and soybeans were inoculated when 70 days old. Crimson, red, and Ladino clover were inoculated when approximately 100 days old. Isolations were made from peas and beans 20 days after inoculation and from alfalfa and the clovers 35 days after inoculation, to determine if the fungus were present in the stems or to confirm visible symptoms. Although the fungus was reisolated from some stems of all species inoculated, typical cankers were observed only on snapbean, alfalfa, crimson clover, and soybean (Table 2). Atypical lesions were observed near the point of inoculation on 1 Ladino and 1 sweetclover stem. No lesions were observed on red clover in this test, but 2 stems died and the fungus was recovered from these stems upon isolation. Garden peas showed no visible symptoms of any kind.

Twenty alfalfa and 18 red clover stems were inoculated with mycelium in the field. At the time of inoculation, the alfalfa was the second growth of the second year and red clover was the first year's growth. Two alfalfa and 1 red clover stem were killed above the point of inoculation. The inoculated stems were brought in from the field in the fall and placed upright in beakers containing ¼–½ in. water, covered with a bell jar, and stored in the laboratory according to the method used by Luttrell (16) to produce perithecia on soybean stems infected with *D. phaseolorum* var. *sojae*. After 3 weeks, the stems were examined under a stereoscopic microscope.

TABLE 2.—*Reaction of various legumes to mycelial inoculation with Diaporthe phaseolorum var. caulivora in the greenhouse*

Crop	No. of stems inoculated	No. of stems infected[a]	No. of cankers formed	No. of stems killed
Garden pea	19	3	0	0
Snap bean	8	8	8	4
Crimson clover	10	7	3	2
Ladino clover	10	2	0	0
Red clover	10	2	0	2
Alfalfa	14	6	6	5
White sweetclover	16	10	0	0
Soybean	16	16	16	16

[a] Infection based on the recovery of the pathogen upon reisolation. Inoculations were made by inserting mycelial-infested toothpicks into the lower portion of the stems.

Perithecia were found on all and were very numerous on several alfalfa stems (Fig. 3). A few perithecia were observed on 4 red clover stems. No pycnidia or conidia were found on either host. These results show that the fungus has the ability to infect some leguminous hosts other than soybeans and under proper conditions of temperature and humidity the sexual stage can be produced. It is possible that some legume species may serve as reservoirs of inoculum for soybeans, although natural infection of these species has not been reported.

Varietal reaction to stem canker.—Differences in varietal reaction to stem canker have been observed during disease surveys in Minnesota, with Blackhawk having the highest incidence of the disease. It has been found on Renville and other varieties recently released or introduced, but the acreage of these varieties is too small to compare them with Blackhawk, which is grown extensively. The spread of stem canker over the soybean area in Minnesota appeared to coincide with the spread of the Blackhawk variety. The writer has seen no stem canker in commercial fields that he could definitely identify as Capital, Ottawa Mandarin, or Flambeau varieties.

It has been suggested that early varieties, such as Flambeau and the slightly later variety Ottawa Mandarin, escape because of their early maturity. It has also been suggested that infection occurs through the leaf or petiole, from which the fungus moves into the stem; and in those varieties in which the lower petioles abscise early, the infected petioles are shed before the organism reaches the stem. The 3 varieties mentioned above shed their lower leaves early, whereas the leaves of Blackhawk do not normally drop off until late summer.

All varieties thus far tested are susceptible to infection when mycelium of the fungus is introduced into the stem; however, some varieties appear to be susceptible to a lesser degree than others. Crall and coworkers reported 15 lines more resistant than Lincoln in Iowa (2).

In 1954, an experiment was designed to study varietal reaction to stem canker under natural field conditions. Sixteen varieties, including 9 recommended to be grown in Minnesota, were planted in a field where 5 per cent of the plants were killed by stem canker the previous year. The field was laid out in plots of single rows 12 ft. long spaced 36 in. apart. All varieties were randomized within each replicate and replicated 5 times. The soybeans were seeded at the regular planting time, May 22.

Very few plants were killed prematurely by stem canker. Evidently conditions were unfavorable for natural early infection during the growing season, as also appeared to be the case in commercial fields of soybeans in the state. Infection, as judged by the presence of cankers, appeared very late in September and in early October. At this time the plants were becoming quite mature, and the infected plants did not show the sudden-death symptoms that are observed in infected plants in a more vigorous growing condition. During most of September there was considerable and continuous wet and cool weather.

The average percentage of infected plants in the varieties tested ranged from 0 to 7.7, and there was no statistically significant difference between any 2 means. Of the varieties recommended to be grown in Minnesota, Renville, Grant, Blackhawk, Chippewa, Capital, Norchief, and Harosoy contained 7.7, 5.3, 5.0, 4.6, 2.9, 2.3, and 1.3 per cent infected plants, respectively, whereas no visible symptoms appeared on Flambeau and Ottawa Mandarin. These varieties were mature when symptoms appeared on other varieties. No symptoms appeared on Pridesoy 11, a variety not recommended in Minnesota and as late maturing as any of the varieties tested. Further testing is needed to determine if this variety is resistant or if it merely escaped infection in this trial.

Although infection at this stage of growth may have no important effect on yield, it may be important in providing inoculum for ensuing years. It is impossible to say at this time if there is a correlation between the amount of late infection in different varieties and the degree of susceptibility of those varieties at a younger age, when considerably more damage may be done by the fungus.

Discussion.—Stem canker of soybeans is a destructive disease in the central and southern part of the cornbelt and was first reported in Minnesota in 1951 (15). Since that time, the highest prevalence observed in Minnesota was 7 per cent in a few fields whereas in the central and southern cornbelt states the prevalence has attained 80 to 100 per cent on susceptible varieties in some fields and experimental plots.

Blackhawk has been the most susceptible variety widely grown in Minnesota. The spread of stem canker coincided with the increased use of this variety; however, the prevalence of stem canker in fields of susceptible varieties has been relatively low in Minnesota compared to that in the areas farther south. In these and other experiments, it was found that the highest percentage of infection occurred when plants were inoculated when 80 or more days old, and results indicate that infections occurring at this time do relatively less damage to plants than do earlier infections. The early maturing varieties in this area are approaching maturity 80 to 90 days after planting. In contrast, the later maturing varieties grown farther south have a longer time to maturity after they have reached the age of 80 to 90 days. This may account, at least in part, for the lower incidence of the disease in Minnesota.

The question of the manner in which natural infections occur has not been answered by these studies; however, subsequent to these investigations Crall (8) reported that infections occur in the blade and petiolar portions of the leaves and that the usual means of entry of the stem canker fungus was at the base of the petioles.

Attempts to produce typical symptoms by applying conidia or ascospores to any portions of the plants at different ages and under different conditions were unsuccessful. The fungus entered the plant tissue, however, as was evidenced by the fact that it was recovered from stem tissue of many inoculated plants. Applying inoculum to autoclaved or field soil, into which a susceptible variety was seeded, failed to produce typical symptoms in the plants. Inserting toothpicks infested with mycelium of the fungus directly into the stems through the leaf scars gave a very high percentage of plants with stem canker symptoms.

It is highly possible that the stem canker fungus is endemic is some of our soils and that infection occurs under certain peculiar conditions of which we are as yet unaware. There is evidence for this in the fact that there is much variation in the amount of infection in the same field or area seeded to the same susceptible variety from season to season. In a field at St. Paul, stem canker appeared in plants grown in a field in which no soybeans had been grown previously. Five per cent of the plants were killed by stem canker. The following season, very few plants of susceptible varieties were killed by the fungus; in a nearby field in whcih soybeans had been grown for 13 consecutive years, no natural stem canker infections have been found. In 1952 and 1953, stem canker was found in all fields examined in Minnesota that were seeded to known susceptible varieties, with as many as 7 per cent of the plants killed in some fields. In 1954, no more than trace amounts of stem canker were found in any of the fields examined.

Experimental results have shown that a small percentage of the seeds from infected plants become infected with the fungus and that infected seeds do not produce plants. It therefore appears that infected

seeds are not directly responsible for plant infection.

Plants of a number of legume species became infected when their stems were inoculated with mycelium of the stem-canker fungus. In a moist chamber, the fungus produced abundant perithecia with ascospores on infected alfalfa stems and, to a lesser degree, on red clover stems. Therefore, it is possible that plants of these species may become infected and serve as a source of inoculum for soybean infection, although this fungus has not been reported as occurring in nature on these species.

Pycnidia with viable conidia were produced on autoclaved plant stems of a number of plant species. Although no typical symptoms appeared, conidia caused a higher percentage of infection than did ascospores, when infection was determined by recovery of the fungus from stems of inoculated plants. It is possible that conidia, although unobserved in nature, may play a role in natural infection of soybean plants, providing infection is caused by spores.

Inoculating 70-day-old soybean plants with mycelium of the fungus caused expression of symptoms in 67 per cent of the plants and a reduction in yield of 10.1 bushels per acre. This confirms, on an experimental basis, the estimates of losses made by various investigators in the field and points to the need for further studies on the nature of infection and methods of inoculation for screening breeders' materials for resistance to stem canker to facilitate the breeding of varieties resistant to this disease.

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A LEAF AND TWIG DISEASE OF ENGLISH HOLLY CAUSED BY PHYTOPHTHORA ILICIS N. SP.[1]

Ivan W. Buddenhagen and Roy A. Young[2]

SUMMARY

A serious leaf and twig disease of English holly in Oregon and Washington previously ascribed to 2 different organisms, *Boydia insculpta* and *Phomopsis crustosa*, is caused by an undescribed species of *Phytophthora*. The disease is characterized by black leaf spotting, defoliation, twig dieback, and the development of black stem cankers. Defoliation was shown to be caused by ethylene evolved from infected leaf tissue. The disease develops during cool moist periods from October to May; the pathogen is inactive in the summer months. Sporangia are produced on the lower surface of infected leaves, and oospores are frequently produced in leaf and twig tissue. The pathogen is a low-temperature species but is separable from *P. hibernalis*, *P. syringae*, and other low-temperature species of *Phytophthora*. *Phytophthora ilicis* n. sp. is proposed.

English holly, *Ilex aquifolium* L., is grown extensively in the Pacific Northwest for the production of cut holly and young trees. A serious leaf and twig disease previously ascribed to 2 different organisms occurs on holly in Oregon and Washington. *Boydia insculpta* sensu Grove was first reported on holly in England in 1921 (7), and Boydia canker of holly in Oregon and Washington was reported on 2 occasions (13, 18). *Phomopsis crustosa* (Sacc.) Bomm. & Rouss. was reported in 1935 (12) to be the cause of brown stem cankers and defoliation of holly in Oregon. These and other reports of fungi pathogenic on English holly were not substantiated by inoculation trials. One organism, *Thielavia basicola* (Berk. & Br.) Zopf, the cause of holly graft failure, has been proved to be pathogenic on English holly (9). The present study, undertaken in 1953–54 to clarify the disease situation on English holly in Oregon, has shown that an undescribed species of *Phytophthora* causes the leaf and twig disease previously attributed to other organisms. Preliminary reports of the disease have already appeared (1, 2, 3).

EXPERIMENTAL METHODS AND RESULTS.—*Media.*—Freshly prepared 2 per cent potato-dextrose agar (PDA) was used for isolation plates and stock cultures; various other media were employed for specific purposes. Among these were Leonian's malt-extract agar (MEA) (10), Petri's mineral solution (17), and the following modified media: bean agar—20 g dextrose, 20 g Bacto-agar, and bean diffusate (prepared by soaking 100 g of dried white beans in hot water for 3 hours) to make 1 liter; milk agar—20 g Bacto-agar, 10 ml whole milk or 2.1 g dried nonfat milk solids, and water to make 1 liter; pea broth—prepared by boiling 150 g of dried whole peas for 30 minutes in 1 liter of distilled water and straining the diffusate through cheesecloth. The pea broth was used to induce sporulation.

Isolation.—Plant parts from which isolations were to be made were washed in tap water, surface-sterilized in 20 per cent Clorox (5.25 per cent sodium hypochlorite) for 1 minute, and washed in sterile distilled water; then, sections were taken from the edge of the diseased areas and planted on PDA in petri plates. Isolations from large stem cankers were made by first inoculating healthy holly stems with the canker tissue and then culturing from the resulting lesions.

Six hundred isolation plates were made from April, 1953, to June, 1954. A great variety of organisms was obtained, but none were obtained consistently until November when a species of *Phytophthora* was first isolated. This Phytophthora was readily isolated from active stem cankers and leaf spots during the winter and spring. If the tissue had been dead for any length of time, secondary fungi or bacteria were recovered instead of the Phytophthora. *Boydia insculpta*, *Phomopsis crustosa*, *Fusarium* spp., and bacteria were most commonly isolated in such cases.

Inoculation.—Stem inoculations usually were made on potted plants; in a few instances they were made on orchard trees by slitting the cortex longitudinally for ½–1 in. and inserting a small portion of mycelium and substrate under the flap made by the slit. The slit was then closed, and absorbent cotton or cheesecloth was placed around the inoculated stem and wet with sterile distilled water. Leaves were inoculated by making 5 needle scratches ¼ in. long on the lower surface and placing a small amount of mycelium and substrate on the scratched surface. Small plants were kept in a humid chamber where the temperature varied from 16° to 22°C. Modifications involving spore suspension sprays or injections were used with several organisms.

All inoculations with the holly Phytophthora were positive except those made on roots (Table 1). This pathogen was readily reisolated from the leaf spots or stem cankers that resulted from inoculations. No symptoms were induced by any of the other organisms except following 1 inoculation made with a hypo-

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TABLE 1.—*Results of inoculations of Ilex aquifolium with various organisms isolated from diseased holly*

Organism	Number of plants infected when indicated plant part was inoculated[a]			
	Stem	Leaf	Berry	Roots
Boydia insculpta	0/75	0/25		
Phomopsis crustosa	1/30	0/10		
Fusarium spp.	0/30	0/10		
Diplodia sp.	0/20	0/10		
Phoma sp.	0/20	0/10		
Nectria galligena	0/10	0/10		
Bacterial isolates	0/20	0/20		
Holly Phytophthora	30/30	50/50	5/5	0/5

[a] Denominator is the number of inoculations attempted; numerator is the number of plants that became infected.

dermic injection of spores of *Phomopsis crustosa*. The 1 positive inoculation indicated that *P. crustosa* may be slightly pathogenic to holly under certain conditions; however, the report (12) of its pathogenicity was based upon the presence of pycnidia in small cankers and upon defoliation of trees containing such cankers. Neither of these symptoms can be relied upon since pycnidia of Phomopsis and fruiting bodies of other fungi are commonly found in old cankers caused by Phytophthora, and defoliation is a symptom of leaf infection by Phytophthora.

The negative results of inoculations with *Boydia insculpta* prove that this organism is not a primary pathogen. Isolation and inoculation results indicate that the "Boydia cankers" reported in the literature were probably Phytophthora cankers in which the secondary organism, *B. insculpta*, was fruiting. This fungus was commonly found fruiting in old black cankers and was occasionally isolated from walled-off cankers. It was found in 10 orchards in Oregon and was especially prevalent in orchards containing cankers and dieback proved to be due to Phytophthora. Phytophthora alone was recovered from a series of greenhouse inoculations made concurrently with *B. insculpta* and Phytophthora, and the former did not fruit in the resulting cankers. Inoculations made in the field with Phytophthora, however, were later invaded by *B. insculpta*.

The Phytophthora disease.—The Phytophthora disease has been found in orchards scattered throughout the holly-growing area of Oregon and in 4 localities in Washington; however, many orchards in Oregon are free of the disease, and the disease is not of economic importance at present in most major holly plantings. Along the coast, where the climate is more humid than in inland areas, orchards are more severely affected. In some of these coastal orchards the disease appears to have been present for many years. A herbarium specimen collected 20 years ago from the Astoria area was found to contain oospores.

The Phytophthora-induced symptom complex consists of leaf spots, defoliation, twig dieback, berry infection, and limb and trunk cankers. Both orchard trees and young holly plants in beds may be affected. The Phytophthora disease develops during the rainy cool period of the year, usually from October to May, and is visibly checked during dry periods in the winter and is completely inactive during the summer.

Black leaf spots (Fig. 1, A) are the first symptoms to appear in the fall after cool rainy weather has begun. The leaf spots usually appear first at the periphery of the leaves on the lower parts of the trees. Defoliation frequently follows spotting, even when the spots are no more than ¼ in. in diameter. Subsequent infection may progress directly up the tree, producing vertical shafts or pyramids of defoliation and leaf spotting. In severe cases, even large trees may become completely defoliated except for a few twigs at the tops of the trees (Fig. 1, B). Inoculum for leaf spot infection is provided by sporangia borne on sporangiophores that emerge through stomata on the lower surface of the leaf. Water droplets hanging from leaf spines of infected leaves during rainy weather have been observed to contain zoospores. Wind whipping of the leaves occurs frequently, and the resulting spine punctures offer ideal openings for infection.

Twig infection follows leaf spot development and continues throughout the winter. That twig infection may occur through newly exposed leaf scars was shown by inoculation. Since holly is harvested in November and December and trees are often pruned additionally after harvesting, pruning wounds also may offer entry points for infection. That such pruning wounds can provide entry for the pathogen was demonstrated experimentally by wounding twigs in a severely affected orchard and isolating the pathogen from the resulting cankers. Large stems may become infected by growth of the pathogen through small twigs and into the stem. Active stem cankers are black, seldom more than 5 in. in length, and frequently are only 1–2 in. long (Fig. 1, C). Cankers frequently become ringed with tan cork tissue and turn lighter in color when inactive for some time. Large limb cankers (Fig. 1, D) and trunk cankers also occur. Trunk cankers have been found only when they were old and walled off, and Phytophthora was not isolated from them.

Infection of ripe berries occurs in late winter and in spring. Girdling of the stem beneath the infected berries may follow (Fig. 1, F) and result in death of the terminal portion of the stem. The leaves beyond the stem girdle first become purple, then tan, and remain on the stem, producing a flagging effect.

Oospores are formed in leaf spots and in the cortex of dead twigs (Fig. 1, E). It is postulated that the oospores provide the oversummering stage of the fungus and that their germination in the fall provides the inoculum for the initial leaf spotting.

Defoliation caused by ethylene.—Ethylene was suspected to be the cause of the defoliation, and 2 experiments were conducted to test this possibility. Inoculated leaves were placed in a sealed 2-quart jar containing a healthy holly twig in a vial of water. As control, a similar jar was set up with scratched but noninoculated leaves in the bottom of the jar, and a

healthy holly twig was placed in a vial of water within the jar. Four replications were used. On the sixth day, the spots were ¼–½ in. in diameter on the inoculated leaves; on the seventh day, the twigs in the jar containing the spotted leaves became defoliated. No defoliation had occurred in the control jars after 21 days, when the experiment was terminated. Defoliation occurred on the seventh and eighth days when 5 or 10 inoculated leaves were used and on the tenth day when only 1 inoculated leaf was used.

A second similar experiment was set up with marigold plants as biological indicators sensitive to the presence of ethylene. This method relies on the epinastic response of marigold plants to minute quantities of

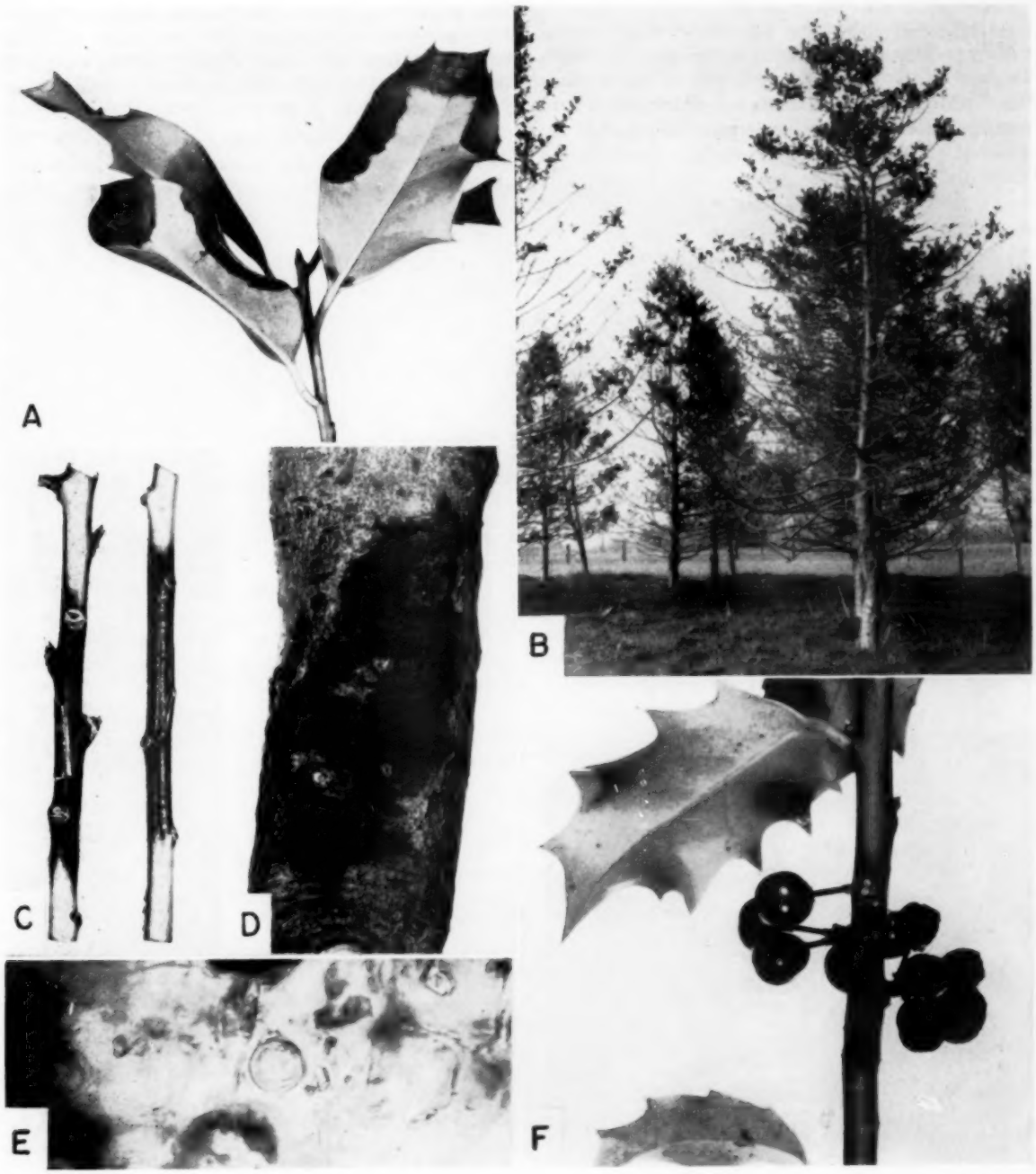

FIG. 1. Phytophthora disease of English Holly. A) Black marginal spots on leaves. B) Partially defoliated orchard trees. C) Active black cankers on 2-year-old stems. D) Canker on 1½-in. limb. E) Oospore in stem cortex. F) Stem canker beneath infected berry cluster.

ethylene (5). Nine inoculated leaves with spots ½ in. in diameter were placed in each of 3 jars with 2 marigold plants. Control jars contained marigold plants and 9 scratched but noninoculated leaves. After 20 hours, the marigold plants enclosed with the inoculated leaves showed epinasty whereas the controls did not (Fig. 2). These 2 experiments demonstrated that the defoliation associated with Phytophthora infection is induced by ethylene gas.

THE PATHOGEN.—*Morphology.*—Mycelial colonies of the holly pathogen on PDA were irregular and layered, with most of the growth submerged in the medium (Fig. 3, A). Sparse aerial hyphae developed toward the center of the colony at low temperature and at the periphery of old cultures. Sporangia were formed abundantly on the lower surface of infected holly leaves. They were not formed on PDA but were produced sparsely on 2 per cent milk agar after 7–10 days and abundantly on washed mycelial mats transferred from 7-day pea-broth cultures (20°C) to sterile distilled water and incubated for 3-5 days at 20°C.

Sporangia, borne singly or on sympodial sporangiophores, were ovoid to obpyriform with shallow apical thickenings (refringent region) and no papillae (nipple-like apex) (Fig. 3, B). At the beginning of germination by germ tube, sporangia appeared papillate. Germination was usually by germ tube at 20°C and by zoospores at 15°C and lower temperatures. Persistent pedicels 5–15 μ long usually remained attached to the sporangia. Sporangia borne on holly leaves ranged from 18–30 × 30–50 μ and averaged 24 × 39 μ. Those borne in distilled water, after pea broth, averaged 18 × 26 μ; in Petri's mineral solution, they ranged widely around an average of 29 × 39 μ.

Oogonia and oospores borne in holly leaves and twigs averaged 21 μ and 18μ, respectively. They were also produced on 1 per cent milk agar and bean agar after 5–8 days, on PDA and MEA after 3–6 weeks (rare), and in sterile distilled water within 4 days after mycelia were transferred from pea broth. Oogonia and oospores on bean agar averaged 27 μ (extremes 25 and 32 μ) and 21 μ (extremes 18 and 25 μ), respectively. Those in sterile distilled water, after pea broth, averaged 25 μ (extremes 17 and 28 μ) and 20 μ (extremes 15 and 23 μ), respectively (Fig. 3, C). The oogonial stalk was either funnel shaped or narrow with a spherical oogonium. The oogonial wall was translucent and the oospore slightly yellow, especially in bean agar. All antheridia observed were amphigynous, averaging 13 × 11 μ.

Occasionally among oogonia, spherical bodies (17–22 μ) were observed that might be classed as chlamydospores or as oogonia that failed to contact antheridia.

Temperature relations.—The holly Phytophthora was grown on various solid media at 5°, 10°, 15°, 20°, 25°, and 27°C. On PDA the average diameters of colonies on 4 plates maintained at these temperatures for 7 days were 3.0, 17.1, 31.0, 33.5, trace, and 0.0 mm, respectively.

Sporangia and oospores were formed most rapidly at 20°C and progressively less rapidly at 15° and 10°C. The increase of size of leaf spots was affected similarly by temperature. Inoculated leaves showed no spotting after 10 days at 5° and 25°C, whereas spot size increased with increase in temperature from 10° to 20° (Fig. 3, D).

Pathogenicity.—Since the holly Phytophthora, *P. hibernalis* Carne, and *P. syringae* Kleb. are close taxonomically, the pathogenicities of the 3 on holly and the first 2 on citrus were compared. Stems and leaves of young holly plants were inoculated in the greenhouse, and excised holly and citrus leaves were inoculated in the laboratory.

The holly Phytophthora was pathogenic on holly leaves and stems but did not invade excised citrus leaves (Table 2). *P. syringae* and *P. hibernalis* were not pathogenic to holly.

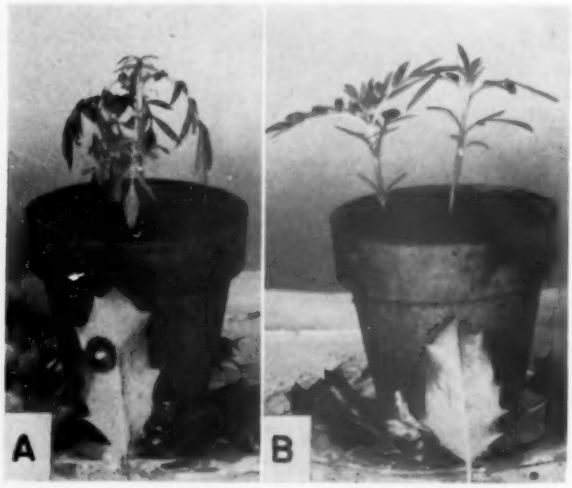

FIG. 2. Epinastic response of marigold plants to ethylene produced by diseased holly leaves. A) Response after 20 hours in bell jar with 9 diseased leaves. B) Check plants after similar treatment with 9 healthy leaves.

TABLE 2.—*Comparison of pathogenicity of holly Phytophthora, P. syringae, and P. hibernalis*

Organism[a]	Number of positive infections[b] resulting from inoculation of		
	Holly leaves	Holly stems	Citrus leaves
Holly Phytophthora	5/5	5/5	0/5
P. hibernalis (Calif.)	0/5	0/5	5/5
P. hibernalis ATC 11300	0/5	0/5	
P. hibernalis CBS		0/5	
P. syringae ATC 11301	0/5	0/5	
P. syringae ATC 11302	0/5	0/5	
P. syringae CBS apricot		0/5	
P. syringae CBS orange		0/5	
P. syringae CBS Peth.		0/5	
P. syringae CBS De Bruyn		0/5	

[a] ATC—American Type Culture Collection; CBS—Centraal Bureau voor Schimmel cultures.

[b] Denominator is the number of inoculations; numerator is the number of resulting infections.

DISCUSSION.—*Comparison with other species of Phytophthora.*—First attemps to classify the holly Phytophthora showed that separation within the genus is difficult and that nearly all characters used by taxonomists for *Phytophthora* are variable or unstable.

Characters used to separate one species from a second may be useless in separating a third from a fourth. Even amphigyny and paragyny, earlier thought to be species constant, may both be present in the same isolate of some species. In spite of this confusion, it was felt that an attempt should be made to classify the holly Phytophthora within the present framework of species separation. Such a treatment should facilitate an eventual reworking of the genus in accordance with modern genetic concepts.

The low maximum temperature at which growth will occur, below 25°C, places the holly Phytophthora with the other low-temperature-maximum species, which in 1931 included *P. infestans* (Mont.) d By., *P. thalictri* Wilson & Davis, *P. phaseoli* Thaxter, *P. syringae*, and *P. hibernalis* (15). Several of these species, however, can grow slightly at 25°C in suitable media.

The holly Phytophthora makes visible growth on malt-extract agar after 6 days at 20°C. On this and other characters it can be separated from *P. infestans*, *P. phaseoli*, and *P. thalictri*. The holly Phytophthora thus falls in Leonian's syringae group which in 1934 consisted of *P. syringae*, *P. hibernalis*, and *P. porri* (11). Species of *Phytophthora* named since 1934 that may be included in this group because of low tempera-

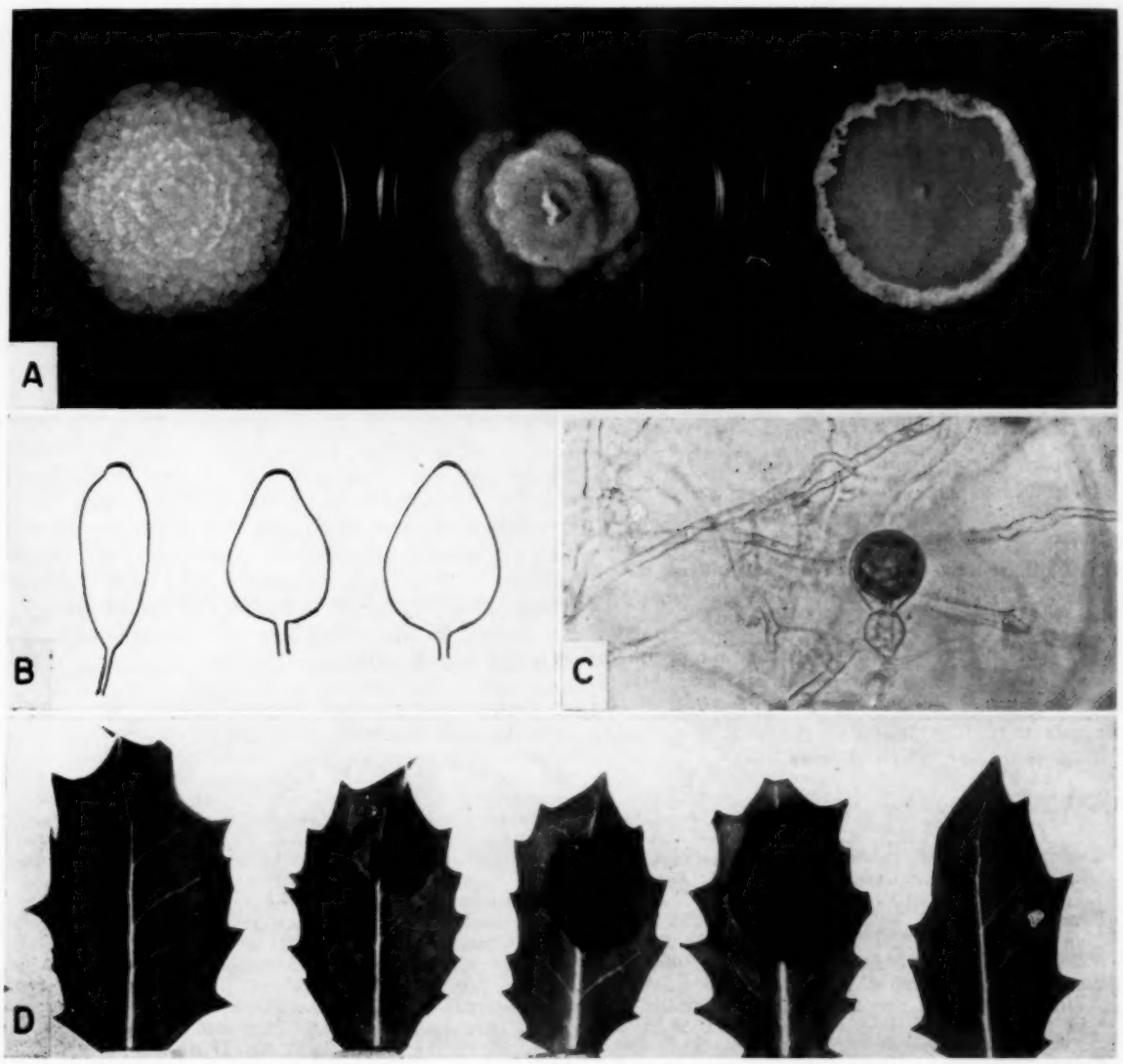

FIG. 3. A) *Phytophthora syringae* (left), *P. ilicis* (middle), and *P. hibernalis* (right) after 10 days at 20°C on potato-dextrose agar. B) Typical sporangia of *P. hibernalis* (left), *P. ilicis* (middle) and *P. syringae* (right). C) Oogonium and amphigynous antheridium of *P. ilicis* × 450. D) Effect of temperature on holly leaf spot development. Leaves inoculated with *P. ilicis* and incubated 10 days at 5°, 10°, 15°, 20° and 25°C (left to right).

ture maximums are *P. fragariae* Hickman, *P. primulae* Tomlinson, and *P. lateralis* Tucker & Milbrath. The holly Phytophthora can be distinguished from these species on the basis that *P. fragariae* does not grow on MEA and does form proliferating sporangia (8); *P. primulae* has paragynous antheridia (14); and *P. lateralis* has a higher temperature maximum, is not known to produce oogonia (16), and is pathogenic on *Chamaecyparis* species only.

The holly Phytophthora can also be distinguished from the 3 members of Leonian's syringae group. *P. porri* grows well at 25°C and has much larger oogonia (6). A comparison with *P. syringae* and *P. hibernalis* is made difficult by the confusion of the taxonomic position of *P. hibernalis*, which was originally described by Carne (4) as having mainly amphigynous antheridia but later was reported by Tucker to be predominantly paragynous and synonymous with *P. syringae* (15). Leonian, however, retained *P. hibernalis* as a separate species, since *P. hibernalis* produced antheridia and oogonia when transferred from pea broth to sterile water whereas *P. syringae* failed to do so. The present authors consider *P. syringae* and *P. hibernalis* distinct species, separable by their reactions when transferred from pea broth to water, by their pattern of growth on potato-dextrose agar, and by the fact of the different shapes of their sporangia. In *P. hibernalis*, the sporangia are characteristically long and narrow, often with the widest point at the shoulder, and they have a long persistent pedicel. The sporangia of *P. syringae* and of the holly Phytophthora are ovate or obpyriform; when a pedicel is present, it is shorter than in *P. hibernalis* (Fig. 3, B).

The holly Phytophthora consistently has amphigynous antheridia and can be distinguished from *P. hibernalis* and *P. syringae*, which have predominantly paragynous antheridia, by that character and by pathogenicity differences. The growth pattern of the 3 species on PDA is characteristically different (Fig. 3, A).

Isolates of the holly Phytophthora could be distinguished readily from other species of *Phytophthora*. The organism appears to be a stable, well-defined taxonomic unit. The following binomial is proposed for the new species. **Phytophthora ilicis** sp. nov.

Hyphis primo continuis, maturitate septatis. Coloniis mycelialibus in agare enormibus, laminatus, margine colonae ampliantis submerso; hyphis aerius raris temperatura inferiore. Incrementum maximum 20°C. Sporangiis nonpapillatis, ovatis ad obpyriformes, pedicellatiis perstantibus, singulariter aut sympodialiter nascentibus; in agare cultis raris, sed copiosis in mattis mycelialibus lavatis e solutione nutritia septem dierum 20°C ad aquam distillatum et sterilem translatis et 3–5 days incubatis 20°C; sporangiis Ilex frondibus 18–30 × 30–50 μ, myceliibus ex infusione pisi 18 × 26 μ. Oogonis et oosporis Ilice aegra 21 et 18 μ, myceliibus ex infusione pisi 25 et 20 μ. Antheridiis amphigynis 13 × 11 μ. Chlamydosporis raris.

Habitat, frondes et rami, Ilex aquifolium L., Oregon et Washington, U.S.A.

Hyphae continuous when young, becoming septate with age. Mycelial colonies on potato-dextrose agar irregular, layered, with outer perimeter of enlarging colony submerged in the medium. Sparse aerial hyphae developing at center of colony at low temperature. Maximum growth at approximately 20°C. Sporangia, with persistent pedicels 5–15 μ, borne singly or on sympodially branched sporangiophores, ovoid to obpyriform with shallow apical thickenings and no papillae; sporangia rarely produced on potato-dextrose agar but abundant on lower surface of infected holly leaves and on washed mycelial mats transferred from 7-day pea-broth cultures (20°C) to sterile distilled water and incubated for 3–5 days at 20°C; sporangia on holly leaves 18–30 × 30–50 μ averaging about 24 × 39 μ, in Petri's mineral solution very variable averaging 29 × 39 μ, and on mycelia from pea broth averaging 18 × 26 μ. Oogonia and oospores in diseased holly leaves and twigs averaging 21 and 18 μ, respectively; on mycelia from pea broth averaging 25 μ (extremes 17 and 28 μ) and 20μ (extremes 15 and 23 μ), respectively; oogonia produced rarely on old potato-dextrose agar cultures. Antheridia amphigynous, averaging 13 × 11 μ. Chlamdospores rare.

Habitat in leaves and stems of *Ilex aquifolium* L., Oregon and Washington, U. S. A.

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PHYSIOLOGY, SPECIALIZATION, AND GENETICS OF PUCCINIA SORGHI ON CORN AND OF PUCCINIA PURPUREA ON SORGHUM[1]

P. M. Le Roux[2] and J. G. Dickson[3]

SUMMARY

A study of the physiology of the fungus, specialization of the pathogen, and the genetics of pathogenicity of *Puccinia sorghi* Schw. and some closely related species was initiated in 1951. A self-inhibitor of germination was demonstrated in both urediospores and aeciospores. Environmental factors influenced the dissipation of the inhibitor and spore germination. Tentatively, 8 lines conditioning resistance and 1 susceptible inbred were selected from maize obtained from various locations in the Western Hemisphere to differentiate 15 races or biotype groups of the pathogen. Uredial and aecial clonal lines of the pathogen were used first in differentiating the biotype groups. Urediospore and aeciospore clonal lines were isolated later and their reaction determined on these differentials. Some of these lines were inbred through the aecial hosts (*Oxalis* species), and the resulting aeciospore populations were analyzed for pathogenicity on the 9 provisional differentials and other hosts. Uredia and telia of *Puccinia purpurea* Cke. on *Sorghum* species, produced infection on *Oxalis corniculata* L. on which abundant aecia were produced. An inbred line from *P. purpurea* (Biotype 13) was pathogenic on 4 of the corn differentials. A second line from the same original clone was pathogenic only on sorghum and Sudan grass. One maize variety, Cuzco, has been resistant to all isolates of the pathogens. The preliminary survey on distribution of biotypes of *P. sorghi* indicated that 3 races occurred commonly from south central Mexico to the north central part of the United States. Apparently, general distribution of urediospores of this pathogen over Central North America was similar to that reported for *Puccinia graminis*.

During the past 5 years, corn rust incited by *Puccinia sorghi* Schw. has increased in prevalence and severity in the central United States. Widespread distribution of urediospores and rust development in June have indicated spore showers from more southern areas. The general rust susceptibility of both dent and sweet corn hybrids and the frequent early spread of rust have indicated the potential threat of this rust to one of America's most important grain crops. Niederhauser and Barnes (18) have reported that corn rust and Helminthosporium blight were the most important diseases of corn in the tropical areas of Mexico and Central America. The 2 diseases have increased in importance in the corn belt of the United States and in the large maize areas of South Africa.

Corn rust has received relatively little attention since the investigations of Mains (15, 16, 17). During the past 20 years, corn production has changed from the use of numerous open-pollinated varieties to the use of hybrids. The relatively limited and similar genetic constitution of corn hybrids grown throughout the corn belt of North America and the general rust susceptibility of these hybrids suggested a search for rust-resistance factors and their incorporation into corn hybrids.

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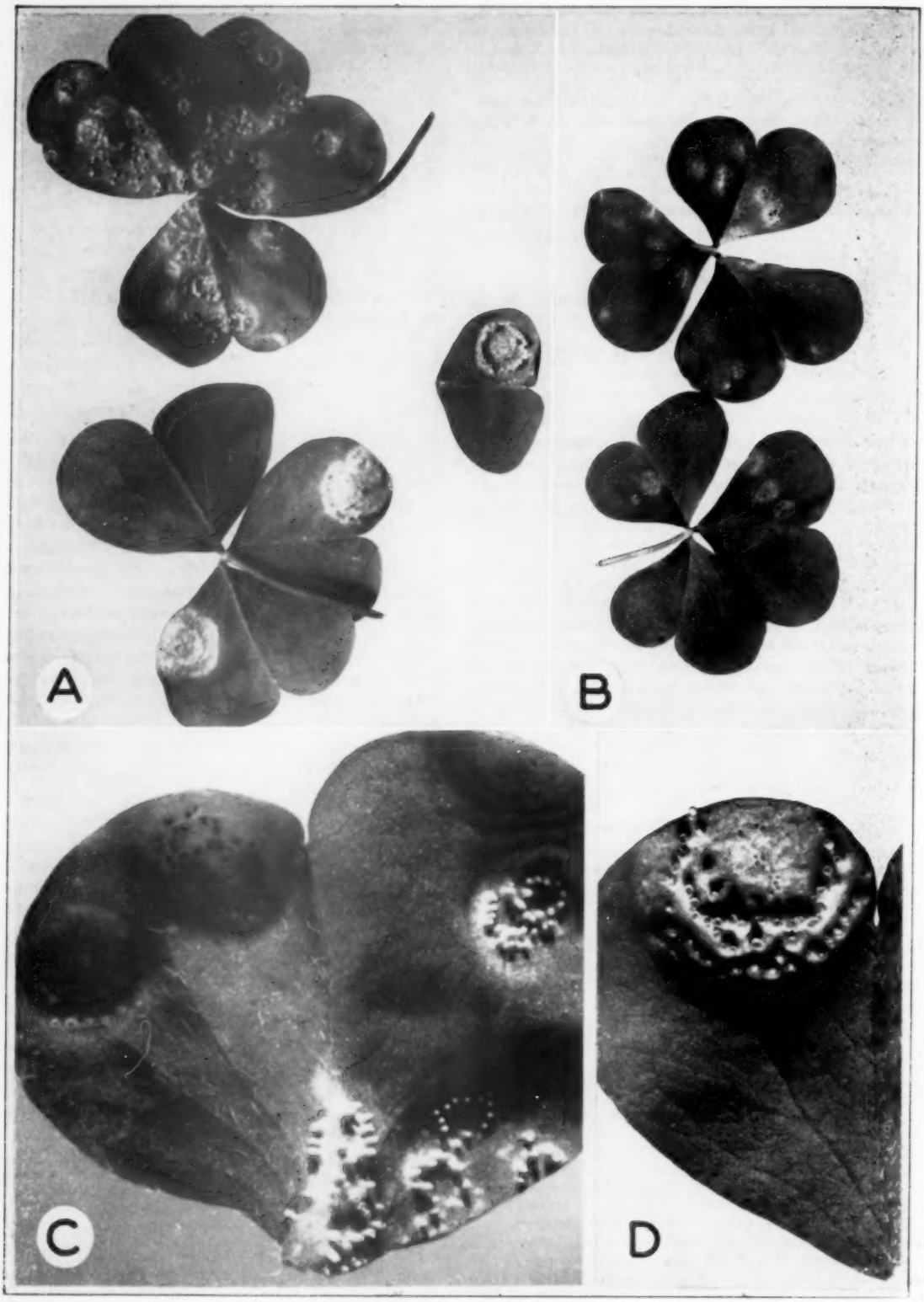


FIG. 1. Aecial development on *Oxalis* species. *Puccinia sorghi* on A) *Oxalis corniculata* var. *corniculata* and B) on *O. europaea* (× 1). C) *P. sorghi* on *O. corniculata*, showing elevated aecia with short aecial cups and serrate peridia (outer edge of leaflet) and depressed aecia with long, serrate peridia (× 5). D) *P. purpurea* on *O. corniculata*, generally with elevated aecia and short serrate peridia (× 5).

The present paper represents the first in a series on the genetics of specialization in *Puccinia sorghi* and related species of which *Oxalis* species are the aecial hosts. This group of rust pathogens, similar in morphology and with such uredial hosts as maize, teosinte, sorghum, and some few additional grasses, appeared especially suitable for a basic study of this type. The large accumulation of data on the genetics of maize, the relatively large number of rust-resistant lines (9, 10), and the standardized methods of breeding offered assurance of suitable differential lines of the host for analysis of specialization in the pathogens. This first report summarizes the preliminary survey on races or biotype groups in *P. sorghi* and the initial study of *Puccinia purpurea* Cke.

Life cycle of Puccinia sorghi and related species.—Arthur (3) apparently was the first to produce the aecial stage of *Puccinia sorghi* on *Oxalis* species, although *Aecidium oxalidis* Thüm. on *O. bowiei* Lindl. was described from the Union of South Africa in 1876. Arthur (3) reported aecial development only on *O. cymosa* Small, which according to Wiegand (27) was *O. europaea* Jord. In investigations on this rust in various countries, Hecke (8), Kellerman (12), Tranzschel (24), Mary Pole Evans (19), Smith (23), Rice (22), Mains (17), and Allen (2) demonstrated infection of 1 or more species of *Oxalis* by the sporidia from teliospores of *P. sorghi*. Some of these investigators also reported specialization of this fungus on the aecial hosts (17). The aecial host occasionally has been found infected in nature (4, 17, 23, 29, 30); however, in the North American corn belt the aecial host apparently has not been an important factor in either the early spread of inoculum to corn or in the natural hybridization of the pathogen.

In the present work, differential response of *Oxalis* species to telial collections of *P. sorghi* was demonstrated. With all isolates studied, pycnia developed on *O. corniculata* L. and its varieties and on *O. europaea* (*O. cymosa* Small and *O. stricta* var. *europaea* Jord.). Aecial development on *O. corniculata* and on *O. europaea* differentiated at least 2 distinct biotype groups of *P. sorghi*. Considerable range occurred in host response and size and number of aecia on the 2 *Oxalis* species (Fig. 1). Neither pycnia nor aecia developed on *O. stricta* L. or on the 20 bulbous species of *Oxalis* tested. *O. corniculata* was the only species found susceptible to *P. purpurea* from sorghum and Sudan grass.

Puccinia oxalidis (Lév.) Diet. & Ell. is distributed widely on the bulbous *Oxalis* species. The junior author has found this Oxalis rust fungus abundant across the central and southeastern United States and Mexico, in the Caribbean Islands, and in several areas of southern Europe. This fungus is reported by Arthur (5) to be widely distributed. Possibly, citron-yellow uredia of *P. oxalidis* on *Oxalis* species have been confused with the aecia of *P. sorghi* in some of the reports of natural occurrence of *P. sorghi*.

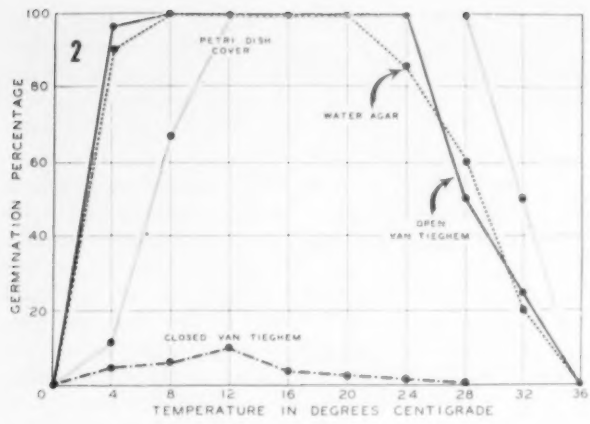
The uredia and telia of *P. sorghi* occur naturally on all varieties of *Zea mays* L. and on *Euchlaena mexicana* Schrad. and *E. perennis* Hitchc. *P. purpurea*, found to have the same aecial host as *P. sorghi*, occurs naturally on *Sorghum vulgare* Pers., var. *sudanse* (Piper) Hitchc., and other varieties and on *S. halepense* (L.) Pers. (11).

The urediospores are long lived and tolerant to heat and desiccation. In mild climates, where the hosts are grown in continuous culture, the pathogen persists in the uredial stage. In more severe climates, a few urediospores survive long periods of dry or cold weather when the leaf tissues of the hosts are protected from disintegration; however, in nature the leaves of the hosts disintegrate soon after maturity. Therefore, the pathogen in most areas is dependent largely upon wind for the spread of urediospores over long distances. This means of distribution in North America is substantiated by the evidence on geographic distribution of biotype groups of the pathogen as reported later.

Physiology of spore germination.—Spore germination of *P. sorghi* was investigated as a guide in selecting the best procedures to use in later studies on pathogenicity. The effect of kind of media, pH of media, aeration, temperature, time, and the conditions under which the spores were stored and germinated were investigated with both urediospores and aeciospores of *P. sorghi*.

The presence of a self-inhibitor of germination in both urediospores and aeciospores imposed an additional complex in spore germination. The physiology of inhibitor formation and dissipation appeared similar to that reported by Allen (1) in urediospores of *Puccinia graminis* f. sp. *tritici* Eriks. & E. Henn. The effect of environment on the synthesis and action of the inhibitor was associated closely with the influence of environment on spore germination (Fig. 2, 3). The inhibitor was active in varying degrees depending upon the specific lot of spores, storage conditions, and period stored. The presence of the inhibitor was determined by germinating the spores in water in closed containers. The inhibitor and its precursor apparently were removed by placing the spores in water in closed containers at 24°C for 12 hours, then collecting and washing the spores and transferring them to another container for germination. Germination of small lots of spores on water agar, in the presence of wet filter paper or in open vessels, removed the major effect of the inhibitor (Fig. 2). In nature, the inhibitor functions only in preventing spore germination while the spores are in the spore-bearing uredium or aecium. Under conditions of restricted air movement in the presence of large quantities of urediospores or heavily rusted corn plants, spores applied to the leaves did not germinate, and therefore infection was not incited. Yarwood (28) demonstrated this phenomenon in the case of bean rust and loosely called it "acquired immunity."

The influence of pH on spore germination was more pronounced with aeciospores than with urediospores



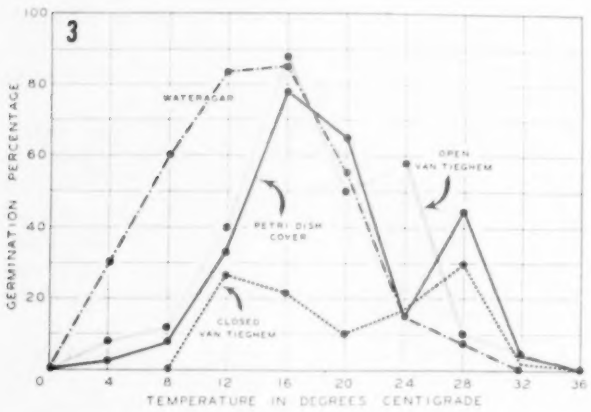


FIG. 2-3. Fig. 2. The relation of temperature, container, and medium to the percentage germination of urediospores of *P. sorghi*.—Fig. 3. The relation of temperature, container, and medium to the percentage germination of aeciospores of *P. sorghi*.

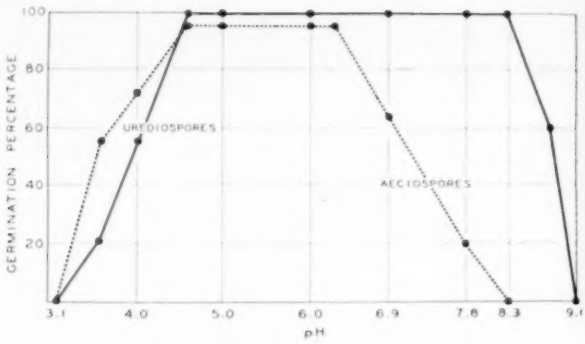


FIG. 4. The influence of pH in a phospate-buffered water agar on the percentage germination of aeciospores and urediospores of *P. sorghi*.

(Fig. 4). The lower limit for optimum germination of both spore types was at pH 4.6. Germination percentages of aeciospores dropped sharply above pH 6.4 and that of urediospores above pH 8.3. Both spore types infect corn leaves, the cell sap of which is buffered naturally within a range of pH 5.4 to 6.4.

The effect of temperature on spore germination also was more pronounced with aeciospores than with urediospores (Fig. 2, 3). The former showed a rather sharp optimum around 12° to 16°C (Fig. 3) and the latter a range from 8° to 28°C, depending upon the container used (Fig. 2). The germination of both aeciospores and urediospores was retarded in closed van Tieghem cells when the spores were not treated previously to remove the inhibitor. The range of temperatures for optimum germination of urediospores was wider than those reported by Mains (14) and Weber (26).

The presence of the germination inhibitor was evident when a mass of spores was confined in a closed container, although spores dispersed widely on the medium or on a leaf surface were not affected greatly by the inhibitor. When single spores were isolated and placed on leaves of susceptible corn inbreds under suitable environments for germination and infection, high infection percentages resulted. Therefore, clonal lines from aeciospores or urediospores were established readily for the various isolates of the pathogens studied.

RUST-RESISTANT CORN FOR DIFFERENTIALS.—Investigations on resistance were started at the point Mains and Rhoades (14, 15, 16, 20, 21) terminated their studies. Only 2 lines studied earlier were available: Golden Glow 208R and sublines of Ball popcorn. The Ball popcorn sublines were not highly resistant and were discarded. GG208R, susceptible to race 3 of the pathogen (17), was retained so that results could be compared with those of the earlier investigations. Seed of this line, which has a single dominant gene pair located on the short arm of chromosome 10 that condition resistance to all physiologic races except race 3 of *P. sorghi* (20, 21), had been maintained by the junior author. Vallega (25) reported 2 biotypes of *P. sorghi* in Argentina; these were differentiated on maize strain 41-3040. A line isolated from a variety obtained from the Cuzco Valley of Equador and Peru was resistant in the present tests to all isolates of the pathogen tested. The 9 lines selected as provisional differentials (Table 1) were found in maize from various locations in the Western Hemisphere. Information pertaining to their isolation and preliminary data on their genetic behavior are given elsewhere (9, 10).

The uredial reaction was determined on the second and third leaves of the seedlings, since the response of the seedling leaf to the pathogen frequently was expressed by a rapid necrosis of the leaf blade (Fig. 5,A). Only 2 reaction types of uredial development were used in classification for rust reaction, although notes were taken in which use was made of the full range of standard designations for uredial reaction (0 to 4 and X); chlorosis and necrosis of surrounding leaf tissue also were noted. Early investigations indicated that many of the intermediate reactions, types 1, 2, and 3, were variable and influenced greatly

TABLE 1.—*Rust reaction in the seedling stage of 8 maize lines to 14 clonal lines of Puccinia sorghi and 1 inbred line of P. purpurea*

Corn line	No.	Rust reaction[a] to lines of *Puccinia sorghi*														
		1	2	3	4	5	6	7	8	9	10	11	12	13[b]	14	15
Cuzco-1	1	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Golden Glow 208-R	246	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R
Oro. V-260-1-2-1	185	R	S	R	R	R	R	R	R	R	S	S	R	R	R	R
B38	214	R	R	R	R	R	R	R	R	S	R	S	S	R	S	R
K148	32	R	R	R	R	R	R	R	R	S	R	S	S	R	S	S
Gto. 29-157A-5-7-1	82	R	R	R	R	R	S	R	S	R	R	R	S	S	R	S
L. I.-27-4-1	178	R	R	S	R	S	R	S	S	R	R	S	R	S	R	R
Gto. 59-272-1-7	155	R	R	R	S	R	R	S	R	R	S	R	R	S	S	S

[a] R = Resistant (0 to 1−); S = Susceptible (1+ to 4).
[b] Culture of *P. purpurea* obtained by inbreeding through the aecial host, *Oxalis* species.

by light, temperature, and other environmental conditions. Throughout the investigations, therefore, the resistant reaction was limited to types 0 to 1− or to the suppression of urediospore formation, and the reaction types 1 to 4 were classified as susceptible (Fig. 5).

The reactions of 9 inbred lines selected as provisional differential hosts were restudied on clonal lines from 38 isolates of *P. sorghi* and *P. purpurea* (Table 1). These 9 lines were designated as provisional differentials pending completion of the determination of the genes conditioning resistance and their recombination into the common genotype B14 (9, 10).

SPECIALIZATION IN P. SORGHI AND P. PURPUREA.—Urediospore and teliospore collections of *P. sorghi* were obtained initially from Mexico, the United States, and Canada. The original uredial collections were increased under isolation on several susceptible inbred lines of maize. Several uredial clones from each collection were isolated and used for inoculation of the 9 tentative differentials. In addition, urediospore clonal lines were isolated, increased, and retested on the differentials. The telial collections were subjected to low temperatures and then used for inoculation under isolation on *O. corniculata* and *O. europaea*. Aeciospores were collected and increased on the susceptible maize lines; then uredial clones were used to inoculate the differentials. Aeciospore clonal lines were used for evaluating the range of specificity in some collections (Fig. 5).

The preliminary survey of the collections of *P. sorghi* made during 1953 and 1954, using the 9 provisional differentials, resulted in the establishment of 15 specialized races or biotype groups (Table 1).

The geographic distribution of the races, based on the isolates studied, indicated the general occurrence of some specialized races through central North America. Races 1, 7, and 10 from Mexico were generally present through the central corn belt of the United States; however, some races found in the corn belt were not collected in Mexico, namely 2, 4, 5, 6, 9, and 15. Race 3, which Mains (13) presumably isolated from somewhere in central United States, was found only in an isolated area in New Mexico. Races 11 and 12 were found only in isolated valleys in Colorado and Wyoming.

Races 8, 14, and 13 were isolated from aeciospores and have not been found in nature. Race 13, an aecial clonal line inbred on its aecial host and pathogenic on corn (Fig. 5, D4), was obtained from teliospores of *P. purpurea* from Sudan grass at Madison. This race has been recovered twice from *P. purpurea*. Inoculations of corn with the original urediospores of these isolates of *P. purpurea* gave no evidence of pathogenicity on corn. Other clonal lines isolated from the same aeciospore population as 13 were pathogenic only on sorghum, Sudan grass, or hybrids between the two. One clonal line recovered from aeciospores from race 4 of *P. sorghi* infected the sorghum 9 × Sudan B hybrid A45 (Fig. 5, D6). Urediospores of the several clonal lines isolated from the 15 biotype groups were increased and placed in storage. Teliospores were produced on the susceptible hosts in the greenhouse, both for preservation of lines and for inoculation of the aecial host.

DISCUSSION.—The present paper represents the first in a series on the study of the genetics of specialization in *P. sorghi* and related species with *Oxalis* species as aecial hosts. This rust group was selected because the large accumulation of data on the genetics of the host, maize, furnished the basic information necessary for locating genes conditioning resistance. The isolation of the several single gene pairs conditioning resistance to specific biotypes of the pathogens and their recombination in a common genotype, inbred B14, in a coordinated research project (9, 10) gives the essential host differentials for the initial analysis of specificity in the several pathogens. Additional genes conditioning rust reaction in maize will be added to this series. A similar series of lines in *Sorghum* species would be desirable, although present interest in the other pathogens relates to the genetic study of lines pathogenic on corn.

The establishment of the biotypes of the pathogen on a gene basis for pathogenicity involves, first, inbreeding and isolation of these genes, and second, recombination and investigation of their mode of inheritance. Flor (6) has stated concerning *Melampsora lini* (Pers.) Lév., "The homozygosity or heterozygosity of the dominant genes is established by selfing the

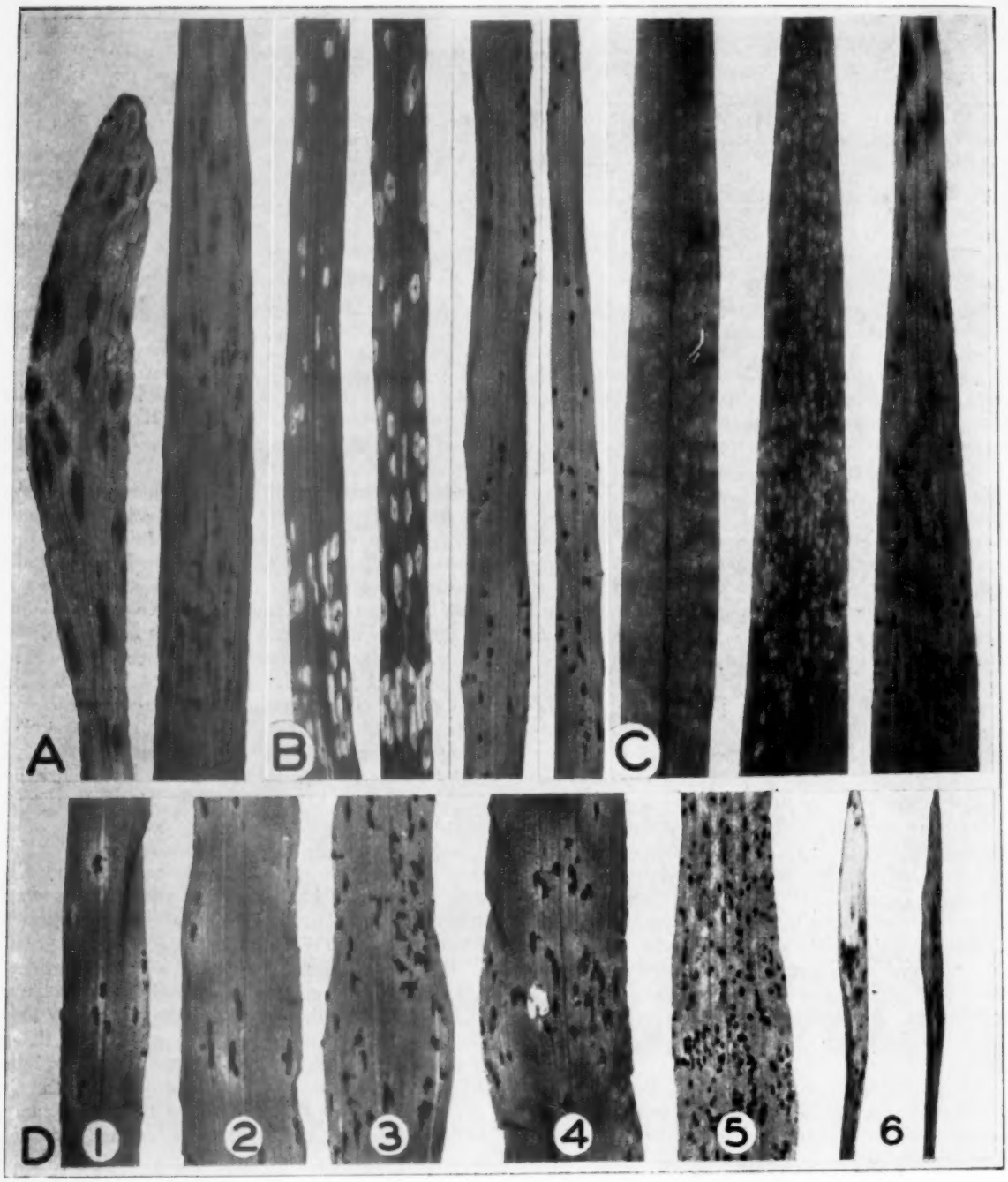


FIG. 5. Rust reaction on seedlings of maize and sorghum. A) Seedling leaf (left) and first leaf (right) of inbred Cap. 63-6-1 (maize), inoculated with clonal line of Race 1 of *P. sorghi*, showing characteristic indefinite reaction with chlorosis and necrosis. B) Reaction of inbred Urq. 30-5-1-1 (maize) to *P. sorghi*. Left pair: Race 5; showing extensive necrosis. Right pair: Race 14; showing susceptible reaction, mostly round uredia. C) Reaction of inbred GG208R (maize) to *P. sorghi*. Left: Race 6; showing necrotic flecks. Center: Race 6; flecks and one type 3 uredium. Right: Race 3; susceptible reaction, mostly elongate uredia. D1–D5) Uredial reaction of inbred L317 of maize to *S. sorghi* and *P. purpurea*. D1–D3) Urediospore clonal lines of Race 1 of *P. sorghi*. D4) Aeciospore clonal line of Race 10 of *P. sorghi*. D5) Aeciospore clonal line 13-18 of *P. purpurea*. D6) Susceptible reaction of sorghum × Sudan grass A45 to aeciospore clonal line Race 4-9 of *P. sorghi*.

uredial clone." The inbreeding of the uredial clonal lines of the maize rust fungus and related pathogens on *Oxalis* species (aecial hosts) and the analyzing for specificity of pathogenicity indicate that frequently more than 1 gene pair for pathogenicity is present in the dicaryotic clone. The apparent suppression of expression of pathogenicity for specific genes in such heterozygous dicaryotic clones, as for example in the clonal line of *P. purpurea*, indicates the importance of selfing in determining the total biotypes present in the culture. The establishment of the biotype on a gene basis for pathogenicity, therefore, involves not only genetic analysis through inbreeding, but also adequate tester host lines with single genes or gene linkages conditioning rust reaction. Flor (6) describes this reaction as the result of specific genes in the host interacting with specific genes in the parasite. In dealing with obligate parasites and with the present inadequate knowledge of parasitism, this perhaps is too simplified an explanation of the reaction. Present evidence indicates that the reactions between host and pathogen are complex and that any list of host differentials and biotypes of the pathogen must be provisional and additive.

The taxonomic implications of the present preliminary study of *P. sorghi* and *P. purpurea* are interesting. Apparently, these fungi have a common aecial host, and they both have genes conditioning pathogenicity on the two uredial host groups.

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Culture of the Rust Parasite, Darluca filum.[1] LUCAS CALPOUZOS, T. THEIS, AND CARMEN M. RIVERA BATLLE.[2] Conflicting reports exist in the cultural requirements of *Darluca filum* (Biv. ex. Fr.) Cast., a fungus that parasitizes rust fungi. Some workers[3,4,5] reported that virulent cultures of *D. filum* can be grown on ordinary media, whereas others[6,7] concluded that it is an obligate parasite. In the present study, *D. filum* was isolated from various hosts and transferred to agar media.

Pycnidia of *D. filum* were removed from their host rust-pustules, rinsed in sterile distilled water, and placed on water agar in Petri plates. The spores, which exuded from the pycnidia, were streaked across the agar surface, and the plates were left overnight at a room temperature of 20° to 27°C. The following day germinated spores were transferred singly or in groups to slants of potato-dextrose agar and soybean-extract agar. Colonies of *D. filum* developed slowly on both media from the spore transfers. Numerous pycnidia, 91–117 μ in diameter, soon began to develop, and typical 2-celled viable spores 15–18μ in length and 3–4μ in width were produced in 7–10 days. Individual colonies reached maximum diameters of 7–10 mm in 3 to 4 weeks.

Each attempt to culture *D. filum* was successful and isolations were made from 8 species of rust fungi, each on a different phanerogamic host species. These were *Uromyces leptodermus* Syd. on *Panicum purpurascens* Raddi, *U. phaseoli* (Pers.) Wint. on *Phaseolus vulgaris* L., *Puccinia cynodontis* Lacroix on *Cynodon dactylon* (L.) Pers., *P. levis* (Sacc. & Bizz.) Magn. on *Paspalum plicatulum* Michx., *P. purpurea* Cke. on *Sorghum vulgare* Pers., *P. sorghi* Schw. on *Zea mays* L., *P. polysora* Underw. on *Tripsacum laxum* Nash, and *Kuehneola malvicola* (Speg.) Arth. H[8] on *Hibiscus syriacus* L. These isolates have been maintained in culture 4 months with subcultures made at approximately 2-week intervals.

Changes in the morphology of some cultures occurred after they were kept on agar media for a time. At first, all isolates looked alike, having small, slightly raised colonies crowded with pycnidia and covered with dense aerial mycelium that gave the colony a moist, compact appearance. After 2 months under the same cultural conditions, 3 of the isolates formed a loose, gray-white, aerial mycelium that made the colonies appear fuzzy. This change has persisted in subsequent transfers.

The pathogenicity of the isolate of *D. filum* from *Uromyces leptodermus* was tested to determine if virulence is retained when the fungus is maintained in culture. A dense spore suspension was prepared by shaking a few ml of sterile distilled water, containing a few drops of a 1 per cent Tween 20 solution as a wetting agent, in an 8-day-old culture of *D. filum*. The spore suspension was sprayed on open pustules of Darluca-free *U. leptodermus* on *Panicum purpurascens* by means of a DeVilbiss atomizer. Six plants were tested as follows: 1) 2 were sprayed with *D. filum*, 2) droplets of spore suspension were placed on pustules of 2 other plants, and 3) 2 were left as untreated controls. All plants were incubated in a moist chamber overnight and then transferred to a greenhouse bench. At the time of inoculation, the germination of the spores was tested and found to be 30 per cent.

When the plants were examined 6 days after inoculation, more than 50 Darluca pycnidia were observed in pustules on each treated plant, whereas no pycnidia were found in the pustules on either of the control plants. Thus, Darluca isolated from *U. leptodermus* retained its ability to infest rust pustules of this species after it had been propagated in culture for 4 months.

During the isolation of *D. filum*, many pycnidia were observed, and from each at least 100 spores were tested for percentage germination on water agar. The germination of spores from 121 apparently mature pycnidia from various hosts was as follows: spores from 62 pycnidia showed no germination, 27 pycnidia had 1 to 50 per cent germination, 10 pycnidia had 51 to 70 per cent germination, and 22 pycnidia had 71 to 100 per cent germination. The differences in percentage germination were not attributable to the hosts from which the various isolates were obtained.

The evidence presented supports the reports of Keener[3,4] and Fedorinchik[5] that *D. filum* is a facultative parasite that can retain its virulence in culture and will grow under ordinary laboratory conditions. These results also show that pycnidia that appear to be mature and are selected for cultural studies frequently contain no spores capable of germination on agar. Such failures might explain why certain inves-

[1] In cooperation with the Field Crops Research Branch, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland.

[2] Plant Pathologists and Research Assistant, respectively.

[3] Keener, P. D. 1933. Some characteristics of Darluca in culture. Pa. Acad. Sci. Proc. 7: 130-139.

[4] Keener, P. D. 1934. Biological specialization in Darluca filum. Torrey Bot. Club Bul. 61: 475-490.

[5] Fedorinchik, N. S. 1952. Virulence and effectiveness of a culture of the rust parasite Darluca filum (Biv.) Cast. (In Russian). Microbiology 21: 711-717.

[6] Traylor, J. A. 1940. Hyperparasites attacking rust fungi in Oklahoma. Okla. Acad. Sci. Proc. 20: 57-58.

[7] Chester, K. S. 1946. The cereal rusts. Chronica Botanica Co. Waltham, Mass. 269 p.

[8] Collected by J. A. B. Nolla, Central Igualdad, Puerto Rico, and identified by J. A. Stevenson, National Fungus Collections, U. S. Department of Agriculture, Beltsville, Maryland.

tigators obtained negative results when attempting to culture this fungus.—Federal Experiment Station, Agricultural Research Service, U. S. Department of Agriculture, Mayaguez, Puerto Rico.

Botrytis on Saintpaulia and its Relation to Mite Control.[1] E. S. McDonough and Robert J. McGray. During the course of a number of experiments designed to perfect a control of mites (apparently *Tarsonemus pallidus* Banks), it was observed that elimination of mites also reduced growth of *Botrytis cinerea* Pers. on *Saintpaulia ionantha* Wendl.

In a typical experiment, the miticide di-(*p*-chlorophenyl) methylcarbinol (as Dimite, a 25 per cent preparation furnished by the Sherwin Williams Company) was applied. Thirty-six 3-in. pots of African violets, each of which was heavily infested with mites, were arranged in a random manner in a greenhouse bench. Each plant was separated from the next by a distance of about 1 ft. High humidity prevailed. Watering and spraying was done with water heated slightly above the air temperature. The temperature of the greenhouse fluctuated widely, the average temperature range being from about 50° to 75°F. A 1-quart hand sprayer was used throughout the experiment. A "full-strength spray solution" was made up adding 4 ml of Dimite to 1 gal. of lukewarm water, a "½-strength solution" by adding 2 ml, and a "¼-strength solution" by adding 1 ml. Control plants were sprayed with water only. Leaves were sprayed every 10 days, both on top and underneath; the young growth at the crown was covered thoroughly.

After the third spraying, plants treated differently differed markedly in appearance. All 9 control plants were practically destroyed; they were heavily infested with Tarsonemus, and the dead and dying parts were covered with the fungus *B. cinerea* (Fig. 1). Except for some spray injury, all 27 plants treated with Dimite were in as good a condition as expected of healthy plants grown under the existing conditions. No mites were found on the 9 plants treated with full-strength solutions, and only a few were found on those violets treated with ½- and ¼-strength solutions. As had been previously reported by Pritchard,[2] full-strength solutions injured the violet plants; some curling and folding of the young leaves occurred. The 9 plants sprayed with ½-strength solution showed little phytotoxic effect of the miticide, whereas the 9 plants treated with a ¼-strength solution developed more and better leaves, had better color, and seemed to produce more flowers than did the other treated plants. All plants in the experiment had at least a few dead leaves covered with Botrytis. It was thought that such leaves had been injured by mites before application of the miticide. The presence of Botrytis growth on the leaves of sprayed plants indicated that Dimite had little if any fungicidal effect, although this point seemed to need further investigation.

Fig. 1. An African violet plant showing the effects of mite infestation and growth of Botrytis. The small pale and distorted crown leaves typify mite injury. The conidiophores of the fungus may be seen on the petioles of leaves near the rim of the pot.

Two repetitions of this experiment, performed at different times of the year, gave the same results. After the first 3 treatments, all 3 sets of plants were sprayed at intervals of 30 days; the plants sprayed with ½- and ¼-strength solutions were brought into saleable condition, free of mites and of Botrytis.

The results of these investigations seemed to justify the conclusion that mite injury may be followed by invasion by Botrytis. Although all the heavily infested and unprotected African violets in the experiment (Fig. 1) did become invaded by Botrytis, many mite-infested violets apparently free from invasion by the fungus have been found in a number of greenhouses. No doubt the abundance of conidia and the nature of environmental conditions,[3] as well as the presence of mite injury, were factors predisposing the plants in the experiments to invasion by Botrytis.

Except for the work of Beck and Vaughn, there seems to be little experimental evidence that Botrytis can invade healthy Saintpaulia tissue. These workers placed plants of several varieties of *S. ionantha* in chambers where light intensity and relative humidity were optimum for the growth of the plants. When plants were sprayed with spore suspensions of *B. cinerea*, the blossom-blight phase of the disease became evident 4 days or more after inoculation. The same symptoms also were observed on African violets

[1] This work was supported by a grant from the Holton and Hunkel Company.

[2] Pritchard, A. E. 1949. California greenhouse pests and their control. Calif. Agri. Exp. Sta. Bul. 713, 72 p.

[3] Beck, G. E., and J. R. Vaughn. 1949. Botrytis leaf and blossom blight of Saintpaulia. Phytopathology 39: 1054-1056.

sprayed with spore suspensions of isolates of Botrytis of the cinerea type from peony, tulip, or geranium. No experimentally induced leaf infection was reported.

Various conditions other than that caused by mite infestation would seem to favor growth of Botrytis on Saintpaulia. As has been reported by Beck and Vaughn[3] and by Roberts,[4] it is not uncommon to find the fungus on the underside of the leaf petiole in the proximity of the pot rim. These authors advised the avoidance of mechanical injury in handling the plants. —Biology Department, Marquette University, Milwaukee 3, Wisconsin.

A Natural Occurrence of the Aecial Stage of Puccinia rubigo-vera var. tritici in the United States.[1] M. N. Levine and R. C. Hildreth.[2] In 1921, Jackson and Mains[3] described the aecial stage of *Puccinia rubigo-vera* (DC.) Wint. var. *tritici* (Eriks.) Carl., the causal agent of leaf rust of wheat, on the basis of infections resulting from controlled inoculations in the greenhouse. They concluded that 2 exotic species of meadow rue, *Thalictrum flavum* L. and *T. delavayi* Franchet, were the only really susceptible alternate host species. They also concluded that the 5 native American species of *Thalictrum* were, with rare exceptions, either resistant or immune. No mention was made of the natural occurrence of *P. rubigo-vera* var. *tritici* on any species of *Thalictrum* in the United States, and no claims were made concerning its possible importance. Since 1921 there has been a tendency among rust researchers to assume that North American species of *Thalictrum* are highly resistant or immune and that aecia of *P. rubigo-vera* var. *tritici* do not occur naturally on that continent. It was further generally assumed that any aecia occurring naturally on species of *Thalictrum* were those of some other gramineous rust fungus near *P. rubigo-vera* rather than those of the leaf rust fungus of wheat.

On June 18, 1954, R. O. Bulger, J. W. Butcher, A. H. Larson, and T. H. Stewart collected an aecial infected specimen of *Thalictrum dioicum* L. in Itasca State Park in Minnesota. The specimen was given to the senior author. Transferred to wheat seedlings, the aecial inoculum produced normal uredial infection. The uredial culture was identified as a typical isolate of physiologic race 2 of *P. rubigo-vera* var. *tritici*. Thus, it now seems that aecial infections of this fungus can occur naturally on a native species of meadow rue in the United States, even if only very rarely.

The significance of this discovery is entirely problematical. In fact, this is not the first instance of the association of aecia of *P. rubigo-vera* with *T. dioicum*. Jackson and Mains obtained at least 1 aecial infection in greenhouse experiments but concluded that the infection was abnormal. In other efforts to produce infections on that species, either only pycnia were developed or no infection whatever occurred. The fact that pycnia developed in some cases suggests that the fungus may have been heterothallic and that failure to produce aecia might have been due to the absence of the proper commingling of pycniospores. It must be concluded, however, that natural infections of this leaf rust fungus on native species of *Thalictrum* are exceedingly rare in the United States, otherwise many would have been reported between 1921 and 1954. Infection on Thalictrum by *P. rubigo-vera* var. *tritici* has never been encountered in the hard red winter wheat area of central United States, although a few aecial infections of other grass rust fungi have been seen on *Thalictrum dasycarpum* Fisch. & Lall.[4] Thus, the occurrence of the aecial stage of the leaf rust fungus in nature would not yet seem to be of much importance.

On the contrary, even such rare occurrences may be of more significance than is immediately evident. They might explain the occasional appearance of new physiologic races; or which is still more likely, the succession of new strains or biotypes of well-known and long-established races that have been noted in recent years. These also raise the question of whether Thalictrum is the only aecial host present in the United States. Bryzgalova[5] discovered in 1937 that *Isopyrum fumarioides* L. was the aecial host of the leaf rust fungus of wheat in the Lake Baikal region of eastern Siberia. This weed grows in the wheat fields and produces aecia abundantly in that area. The only species of *Isopyrum* found in the central grain-producing area of the United States is *I. biternatum* (Raf.) T. & G., which Mains[6] was unable to infect with the leaf rust fungus of wheat, although it is known to be susceptible to certain grass rust fungi of the group around *P. rubigo-vera* (DC.) Wint. var. *agropyrina* (Eriks.) Carl. This discussion raises the question of whether the leaf rust fungus of wheat is the same species throughout the world or whether it is composed of 2 or more sub-species or tribes.—U. S.

[4] Roberts, E. 1951. Leaf rot (Botrytis cinerea) of African violets. African Violet Mag. 5: 13.

[1] U. S. Dept. of Agriculture, Agricultural Research Service, Field Crops Research Branch, in cooperation with the Department of Plant Pathology and Botany, University of Minnesota. Paper No. 3456, Scientific Journal Series, Minnesota Agricultural Experiment Station.

[2] Pathologist, Field Crops Research Branch, ARS, U. S. Dept. of Agriculture, and Research Assistant, University of Minnesota.

[3] Jackson, H. S., and E. B. Mains. 1921. Aecial stage of the orange leaf rust of wheat, Puccinia triticina Eriks. Jour. Agr. Res. 22: 151-172.

[4] According to correspondence with C. O. Johnston, Pathologist, Field Crops Research Branch, ARS, U. S. Dept. of Agriculture, Kansas Agricultural Experiment Station, Manhattan, Kansas.

[5] Bryzgalova, V. A. 1937. On a new intermediate host of brown rust of wheat, Puccinia triticina Erikss. (Translated title). Sbor. Trudovpo Zashch. Rast. Vostochn. Sibiri 5: 75-88.

[6] Mains, E. B. 1933. Host specialization in the leaf rust of grasses, Puccinia rubigo-vera. Mich. Acad. Sci. Arts, and Letters. Papers 17: 289-394.

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Presence of Pectic and Cellulolytic Enzymes in Tomato Plants Infected by Pseudomonas solanacearum. AKHTAR HUSAIN AND ARTHUR KELMAN. In an investigation of the mode of pathogenesis of *Pseudomonas solanacearum* E. F. Sm., causal agent of southern bacterial wilt, production of pectic and cellulolytic enzymes by the bacterium in culture was demonstrated.[1] In this study it was also noted that rapid maceration of the basal portions of tomato (*Lycopersicon esculentum* Mill.) cuttings occurred when they were immersed in culture filtrates containing these enzymes. A similar decay of parenchymatous tissue can be observed in succulent host plants infected by this bacterium. Since the determination of the role of enzymes *in vivo* requires evidence of their presence in diseased plants, experiments were designed to determine whether *P. solanacearum* produces polygalacturonase and a cellulolytic enzyme (Cx) in tomato plants. Rapid breakdown of host tissues following infection by *P. solanacearum* has been attributed frequently to the action of secondary bacterial invaders. In order to be certain that the enzymes present in decayed tissues were produced by *P. solanacearum* alone, it was also necessary to determine the degree of success of measures taken to prevent entry of secondary bacteria.

Eight-week-old Rutgers tomato plants were stem-inoculated by means of hypodermic syringe containing a suspension of a highly pathogenic isolate of *P. solanacearum*. The inoculation court was surface sterilized prior to injection of bacteria and then covered with a sterile cotton pad to prevent contamination. After 10 days, inoculated plants showed intermediate to advanced symptoms of bacterial wilt.

To determine whether secondary organisms other than *P. solanacearum* were present in wilted plants, isolations were made 10 days after inoculation from 20 plants showing advanced decay of pith and vascular tissue. Segments of internal tissues were removed from the point of inoculation and at points 2 and 4 cm below the inoculation court. Bacterial suspensions obtained were streaked on a peptone casein-hydrolyzate medium containing 2,3,5-triphenyltetrazolium chloride.[2]

In isolations made in the above manner, bacteria other than *P. solanacearum* were present in 3 of 20 samples taken at the point of inoculation and in 2 of 20 samples at a point 2 cm below the inoculation court. In all instances, however, the relative numbers of contaminating bacteria present were very low (less than 10 per cent of total bacterial population). In each case where secondary bacteria were present, the stem tissues had collapsed in the region of the inoculation, and necrotic areas were evident on the surface of the stem. In isolations from tissues showing advanced decay, colonies of the weakly pathogenic variant form of *P. solanacearum* also were present. The percentage of colonies of the variant type usually decreased as the distance from the point of inoculation increased.

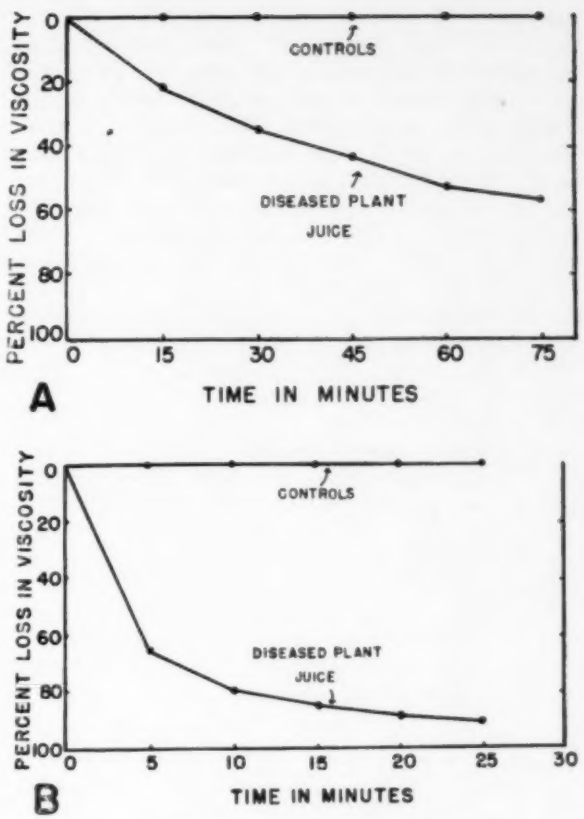

FIG. 1. A) The effect of juice from diseased plants on the loss in viscosity of a 1.2 per cent solution of carboxymethylcellulose as compared with controls (juice from healthy plants and heated juice from diseased plants). B) The effect of juice from diseased plants on the loss in viscosity of a 1.2 per cent solution of sodium polypectate as compared with controls (juice from healthy plants and heated juice from diseased plants).

In order to obviate any possible effects resulting from the growth of contaminating bacteria, any stem segments showing external evidence of tissue collapse and advanced cortical decay were not used in the enzyme assays described below.

Plant juice to be used in enzyme assays was obtained in the following manner. Stems of 20–25 wilted plants were cut into small segments, thoroughly

[1] Husain, A., and A. Kelman. 1956. Mode of pathogenesis of Pseudomonas solanacearum. (Abs.) Phytopathology 46: 16.

[2] Kelman, A. 1954. The relationship of pathogenicity in Pseudomonas solanacearum to colony appearance on a tetrazolium medium. Phytopathology 44: 693-695.

washed in sterile water, and frozen. After thawing, the stems were ground; the juice was expressed through cheesecloth and then centrifuged. The supernatant was passed through a Seitz filter, and the filtrate was used for enzyme assay. Samples of plant juice also were obtained in a similar manner from healthy tomato plants of the same age. Polygalacturonase activity was measured by estimating the loss in viscosity of a 1.2 per cent solution of sodium polypectate at pH 5.5 in size 300 Fenske-Ostwald viscosimeters at 30°C. Cellulase activity was assayed by a similar method with a 1.2 per cent solution of carboxymethylcellulose at pH 5.5 used as substrate. Samples of juice from healthy plants and of heated juice from diseased plants served as controls.

In 3 successive trials, samples of juice obtained from diseased plants showed significant cellulase (Cx) and polygalacturonase activity. In contrast, no enzymatic activity was detected in juice from healthy plants or in heated juice from diseased plants. Representative data from assays for cellulase (Cx) and polygalacturonase are presented in Figure 1.

These data indicate that the breakdown of parenchymatous tissue that characterizes this disease in tomato can be attributed to the action of the above enzymes formed by the bacterium in the host. Furthermore, it appears that decay of tissues can be brought about by the action of *P. solanacearum* in the absence of other secondary bacteria.

The demonstration of these 2 enzymes in diseased plants supports data obtained in earlier studies in which culture filtrates were used. Previously, evidence has been presented indicating that polygalacturonases play a role in wilting caused by *Fusarium oxysporum* f. *lycopersici* (Sacc.) Snyd. & Hans.[3] and by *Verticillium albo-atrum* Reinke & Berth.[4] Presence of such enzymes has not been demonstrated in host plants affected by these wilt pathogens, however, although pectin methyl esterase has been found in the vascular fluid of tomato plants affected by Fusarium wilt.[5] The exact role and importance of pectic and of cellulolytic enzymes in wilt induction by *P. solanacearum* remains to be determined.—Plant Pathology, North Carolina State College, Raleigh, North Carolina.

[3] Gothoskar, S. S., R. P. Scheffer, J. C. Walker, and M. A. Stahmann. 1955. The role of enzymes in the development of Fusarium wilt of tomato. Phytopathology 45: 381-387.

[4] Scheffer, R. P., S. S. Gothoskar, C. F. Pierson, and R. P. Collins. 1956. Physiological aspects of Verticillium wilt. Phytopathology 46: 83-87.

[5] Waggoner, P. E., and A. E. Dimond. 1955. Production and role of extracellular pectic enzymes of Fusarium oxysporum f. lycopersici. Phytopathology 45: 79-87.

LISTING OF BOOKS

PHYTOPATHOLOGY will, from time to time as space becomes available, list titles of books on phytopathological subjects. A brief description of the contents of the books will be included.

NORTHEN, H. T., AND REBECCA T. NORTHEN. 1956. **The complete book of greenhouse gardening.** Ronald Press Co., 15 E. 26th Street, New York 10, N. Y. 353 p. Price $6.50.

Most of this book is devoted to specific information on all phases of growing a wide variety of plants under glass. Emphasis is placed on ornamental plants, but information also is given on vegetables. Practical information and directions are given on the selection of a greenhouse, on its management, on soils and nutrition, on propagation, on plant pests, and on diseases of greenhouse plants.

STAPP, C. 1956. ***Bakterielle Krankheiten.*** **Sorauer Handbuch der Pflanzenkrankheiten.** Part 2. Vol. 2. Paul Parey, Berlin and Hamburg. 567 p. 179 Illus. Price DM 124.

After a short introduction, the author describes the bacteria that have been found infecting 67 host families from the fungi to the Compositae. Briefly, the symptoms, description of the organism, life history, control, and the important citations are given for each bacterium. Fifty-eight pages are devoted to Streptomyces, most of which deal with the ones causing potato scab. The book is remarkably complete in bacterial nomenclature. The printing, paper, and general formation of the volume are excellent.

KAMAT, M. M. 1956. **Introductory plant pathology.** Published by the author at Prakash Publishing House, 503/3 Budhwar Peth, Poona 2, India. Price: Royal size—Rs. 10/-net (inland), 15s (overseas); Special student edition—Rs: 9/-net (14s). 244 p. 365 Illus.

This book supplements the author's earlier publication, *Practical Plant Pathology*, and is intended to complete a course in plant pathology, both theoretical and practical, at the B.Sc. level as sponsored by Indian universities. Although written for Indian students, it should be useful elsewhere. Special emphasis is placed on tropical diseases. Examples and illustrations are drawn, as far as possible, for Indian material. Fifteen of the 28 chapters are devoted to a discussion of events, in natural sequence, that lead to disease. The remaining chapters deal with control, methods of studying plant diseases, taxonomy and nomenclature, and the various groups of plant pathnomenclature, and the various groups of plant pathogens.